

VOL. 42, PART 3

SEPTEMBER 1959

The British Mycological Society

(Recognosce notum, ignotum inspice)

TRANSACTIONS

Edited by

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CAMBRIDGE UNIVERSITY PRESS

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NEW YORK, N.Y. 22

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Enquiries from the U.S.A. should be addressed to the Cambridge University Press American Branch, 32 East 57th Street, New York 22.

Trans. Brit. mycol. Soc. **42** (3), 273–286 (1959).

THE REGULAR GERMINATION OF RESTING SPORES AND SOME OBSERVATIONS ON THE GROWTH REQUIREMENTS OF AN *ENDOgone* SP. CAUSING VESICULAR-ARBUSCULAR MYCORRHIZA

By BARBARA MOSSE

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(With Plates 14–17 and 3 Text-figures)

Aseptically grown apple and strawberry seedlings inoculated with surface-sterilized spores of an *Endogone* sp. have produced typical vesicular-arbuscular mycorrhiza.

A method is described of multiplying the sporocarps of this fungus, and of obtaining a regular supply of aseptic germinated spores which can be used to provide a pure inoculum. Some of the growth requirements of this *Endogone* sp. are discussed.

INTRODUCTION

Endotrophic mycorrhizal infections of the type now generally called vesicular-arbuscular were first clearly described by Dangeard (1900) who gave the generic name of *Rhizophagus* to the fungus concerned. Because Dangeard was the first to recognize that arbuscules and vesicles were growth forms of the same fungus, and because his illustrations were so characteristic and life-like, the name *Rhizophagus* was widely accepted and is still occasionally used (Neill, 1944; Barrett, 1947, 1958). Since, however, no genus or species description of such a fungus exists (Dangeard himself believed that it might belong to the Chytridiales), it has become more usual to refer to the fungi concerned as vesicular-arbuscular endophytes.

The ubiquitous nature of such root infections became apparent during the last fifty years. The extensive surveys of Peyronel (1923–4), Jones (1924), Samuel (1926), Butler (1939), Lihnell (1939), Neill (1944), Winter & Birgel (1953), Strzemska (1953) and others leave little doubt that potentially the roots of most, if not all Angiosperms, growing under a wide range of natural and man-made ecological surroundings, are capable of acting as host to a fungus, or a group of fungi, of almost universal distribution in soils, which when growing within the roots develops the distinctive characteristics of a vesicular-arbuscular endophyte. Visual evidence clearly indicates that the hyphae of this fungus are among the most commonly occurring rhizosphere mycelia, but the fungus never appears on dilution plates or in lists of soil fungi because it has very specialized growth requirements, and in spite of numerous attempts no culture medium has yet been found on which it can be isolated. Not infrequently a limited amount of growth has been obtained from infected

root fragments (Magrou, 1946; Stahl, 1949, and others), or from vesicle-like spores in the soil (Butler, 1939), so that the main growth characters of the mycelium are fairly well known and there is now wide agreement that it probably belongs to the *Phycomycetes*.

A real advance in the understanding of these widespread and often very spectacular root infections (not infrequently more than half the primary cortex of apple and strawberry roots consists of fungus tissue) depends on the ability to culture the fungus, or at least to produce typical infections from pure inocula. During the last decade such a possibility seems to have come within reach, with two reports of the culture of a fungus claimed to cause typical vesicular-arbuscular infections (Barrett, 1947, 1958; Hawker, Harrison, Nicholls & Ham, 1957), and two reports that such infections have been produced by inoculation with surface-sterilized spores (Gerdemann, 1955; Mosse, 1953, 1956). None of these claims has so far been confirmed. The report describing the successful inoculation of aseptically raised seedlings with surface-sterilized spores of an *Endogone* sp. (Mosse, 1956) is open to the criticism that a fairly high proportion of the spores are infected internally with other organisms, that the success of inoculations varied with the condition and source of spores, and that, since no pure two-membered cultures consisting of plant and fungus only were ever achieved, the surface-sterilized spores did not in fact represent a pure inoculum. Some of these objections have now been met and a technique has been developed for obtaining a regular supply of aseptic germinating spores. With the aid of these *Endogone* spores some progress has been made towards establishing in culture a fungus capable of causing typical vesicular-arbuscular infections in a range of plants, and closely resembling in its growth habits the fungus grown by Magrou, Stahl and Butler. Some of the growth requirements of this fungus have been studied in systematic experiments. Since the provision of a pure inoculum opens a wide field for the future investigation of endotrophic mycorrhizal infections, it is perhaps timely to give an account of the techniques used and of the information so far obtained.

PRODUCTION OF SPOROCARPS

The resting spores of the *Endogone* sp. under discussion are formed in sporocarps like those shown in Pl. 14, fig. 1. They have been described in some detail in a previous paper (Mosse, 1956). Such sporocarps were first found attached to the roots of a strawberry plant grown in a glass-house pot in a steam-sterilized compost (John Innes no. 1) and it is now thought that they may have originated from the unsterilized peat that is a component of this compost. While such fructifications occur rarely, if at all, associated with plants grown under field conditions, it has proved simple to ensure a regular and unlimited supply of sporocarps by the following method. Loam and sand are autoclaved separately in pots (15 lb. pressure for 30 min.), covered with newspaper and stored for at least 6 weeks in a normal atmosphere. When ready for use, the two are mixed in the proportions 2 parts loam: 1 part sand, and placed in sterilized pots. Seeds are sown or runners established in the autoclaved soil. When

the plants have grown sufficiently for their roots to form a loose network over the surface of the soil ball, this is gently knocked out of the pot, and thirty to fifty sporocarps are carefully pushed into the soil surface in the vicinity of a growing root. With some care this can be done when the soil is fairly wet without disturbing the roots. The plant is put back into the pot and watered when necessary with fresh tap water. After 4-6 months of vigorous growth, upwards of 2000 new sporocarps will have developed in association with the young roots. When these are carefully soaked out of the soil, they will present an appearance like that shown in Pl. 14, fig. 2. Sporocarps have been multiplied in this way in association with apple, strawberry, onion and barley roots, in plants grown both during the summer months, and, with extra lighting, during the winter. The yield of new sporocarps was most satisfactory with apples and strawberries. The method does not appear to be at all critical and composts consisting of autoclaved soil and peat, and of steam-sterilized soil and peat have also been used successfully. Completely unsterilized soil does not favour the production of new sporocarps, and in one experiment where strawberry runners were grown in autoclaved soil placed in large polythene-lined galvanized iron containers (3 ft. diam. $\times$ 2½ ft. deep) placed out of doors and inoculated with 100 sporocarps each, no new sporocarps were formed. The production of new sporocarps is apparently a response to nutritional conditions and requires a certain density of actively growing roots and an intensity of mycorrhizal infection only achieved after complete or partial suppression of competing soil organisms.

The condition of resting spores at the time of collection is of importance for their subsequent germination. It is therefore necessary to give some information on the development of sporocarps so far as it can be pieced together from observations made during the dissection of many hundreds of sporocarps at various intervals after inoculation. The majority of sporocarps appear to originate as a closely interwoven mass of fine, densely branched hyphae that develop in and around small fragments of a brownish resin-like material, sometimes of discernable plant origin. Occasionally the origin of the hyphal matrix from one repeatedly branched main hypha is evident, (Pl. 14, fig. 3). Within the hyphal matrix, and generally arising from the plexus-like junction of several anastomosing hyphae, the resting spores are formed (Pl. 15, fig. 4), sometimes singly, but more often in a closely packed interconnected group. They quickly enlarge and spores up to 200μ are found with granular multinucleate contents and colourless thin walls, easily penetrated by cotton blue. Even at this early stage the spore contents are usually separated from those of the parent hypha by a basal plug continuous with the inner spore wall. The majority of spores within a sporocarp seem to develop simultaneously and during their rapid swelling in a confined space they often acquire angular outlines. Not infrequently empty collapsed spore walls are found and occasionally spores of the surrounding extra-matrical mycelium also become included in the developing sporocarp. When the spores have nearly reached their full size the inner spore wall begins to thicken and become yellow while the spore contents gradually become highly vacuolated. At first the outer and inner spore walls do not separate easily and on dissection

spores emerge from the sporocarps with a halo of closely attached fine hyphae. Attempts to remove these usually result in rupture of the spore. A more definite separation of the spore walls occurs gradually, and at maturity a slight external pressure, such as that applied when picking up sporocarps with a pair of forceps, is sufficient to extrude the highly vacuolated, clean, shiny, bright-yellow resting spores. The thin papery envelope which is all that remains of the outer spore wall is left behind in the sporocarp. All the evidence suggests that at this stage, which occurs 4-5 months after inoculation and often coincides with the cessation of active vegetative growth in the associated plant, the spores are mature and that at this time natural spore dispersal occurs, possibly by the action of drainage water. When the proportion of sporocarps with mature vacuolated spores is at its maximum (90 % or more), sporocarps intended to provide material suitable for germination should be collected and stored on damp filter-paper at 5° C. After about 6 weeks storage some of the spores will have become dense and opaque looking as their contents change from a transparent, homogeneous, highly vacuolated protoplasm with conspicuous oil droplets to an opaque, particulate, granular structure lacking oil drops or vacuoles. Such spores frequently harbour internal parasites which on germination give rise to a mass of fine, regularly septate hyphae. The majority of spores will still be bright yellow and highly vacuolated, and these are capable of rapid germination and of providing a pure inoculum. After 3 months storage, the proportion of opaque spores increases rapidly. Their walls darken and take on a mottled appearance, owing to the formation of prominent internal wall projections resembling lignitubers (Mosse, 1956). Still later many of the spores lose their contents and only a brittle, dark brown, empty shell remains. Sporocarps left undisturbed in the soil show similar developmental stages though these may occur more slowly, and there is evidence to suggest that the formation and development of sporocarps depend in some measure on the activity of the associated plant. An interesting reversal of the normal process of wall thickening, together with a loss of storage material and degeneration of the spore contents, was observed in transplanted apple seedlings kept over the 1957/8 dormant season. It is hoped to repeat this observation.

GERMINATION OF RESTING SPORES

At certain times, usually in the spring, resting spores placed on damp filter-paper or on water agar will germinate spontaneously giving rise to a coarse aseptate mycelium characteristic of this *Endogone* sp. (Mosse 1954, 1956). Even under the most favourable conditions, such spore germination extends over a period of 2-3 weeks. At other times germination is very erratic and usually below five per cent. Godfrey (1957), studying spore germination in other *Endogone* species with very similar germination processes, has encountered a similar irregularity. Much more regular results can be obtained by the method described below which, in the *Endogone* sp. here described, leads to 80 % or more germination over a period of 3-4 days.

A regular supply of uncontaminated, germinated resting spores depends on three conditions:

(1) A careful selection of chilled resting spores in a suitable state of development, with thick yellow walls and highly vacuolated contents. A successfully germinated spore with typical contents is shown in Pl. 15, fig. 5. It is very important to discard all spores with opaque contents, and the entire sporocarp should be discarded if it contains even one such spore. Immature spores in which separation of exo- and endospore wall is incomplete should also be avoided.

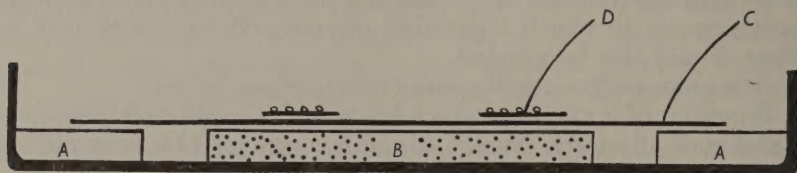
(2) Adequate surface sterilization of the excised spores.

(3) Provision of a stimulus which leads to the rapid and simultaneous germination of all spores. Such a stimulus is provided by some dialysable metabolites produced in soil agar plates.

The following methods have been used to provide these conditions. It is not suggested that the methods are critical and modifications are likely to be just as effective provided the basic principles are observed. Sporocarps are placed on a piece of damp filter-paper in a Petri dish and are dissected with a pair of needles under a dissecting microscope with strong substage illumination. Vacuolated spores appear somewhat translucent, and bright yellow in colour. The excised spores can easily be freed from the remains of the exospore wall by moving them gently across the damp filter-paper. Suitable spores are picked up individually on the tip of a needle and transferred to a sterile watch-glass containing a freshly made solution of 2 % chloramine T + 200 p.p.m. streptomycin and a trace of detergent. Some spores will sink while others float in the sterilant. This is without significance for their future germination, but care must be taken to ensure adequate sterilization of the floating spores. Spores are then transferred to a fresh solution of sterilant, by means of a sterile capillary pipette. After a total sterilization period of about 15 min. (the timing is not critical), the spores are rinsed in two changes of sterile water. They are then placed on small pieces of 'Cellophane', about 0.5 cm. square, that have previously been autoclaved between pieces of damp filter-paper. After this spores are no longer handled individually, but each square with its spores becomes a potential source of pure inoculum. The Cellophane squares are immediately transferred to soil agar plates prepared as follows. A flat metal or glass ring, c. 5 cm. diameter, 0.5 cm. thick and 0.5 cm. high, is placed inside a sterile Petri dish. An outer ring of 0.75 % water agar is poured and allowed to set. Sieved soil (approx. 0.2 g.) is then placed inside the metal ring and 5 ml. of 0.75 % water agar near setting point is poured into the centre and allowed to solidify after gentle agitation of the Petri dish to ensure an even distribution of the soil particles within the ring. When the agar has hardened the metal ring is removed. A previously autoclaved Cellophane disk, c. 7 cm. diameter, is then placed over the agar so that its outer edge rests on the plain agar. The small Cellophane squares carrying the spores are then placed over the soil: agar part of the plate. A cross-section view of such a plate (Text-fig. 1) may help to clarify the procedure.

The arrangement is designed to minimize the risk of contamination of the upper surface of the large Cellophane disk (C), while allowing diffusion from the soil: agar to the spores. The Petri dish is then incubated at 20° C.

After 2 to 4 days, 80–100 % spores will have begun to germinate and 2 days later the Cellophane squares will be covered by a network of coarse, translucent, anastomosing hyphae visible to the naked eye. A typical example is illustrated in Pl. 16, fig. 6. Before being used as inocula, the transparent Cellophane squares can be examined microscopically for the presence of contaminating organisms.



Text-fig. 1. Diagram of soil:agar plate used for germination of *Endogone* spores. A = water agar; B = soil + water agar; C = large 'Cellophane' disk; D = 'Cellophane' square with spores.

Varying the above conditions has provided some clue as to the nature of the stimulatory substance. Germination is not affected by substances diffusing from the Cellophane or the water agar. It is affected somewhat by physical conditions and will be retarded if the Cellophane is too dry. Reversing the Petri dishes so that condensation water collects on the Cellophane helps to counteract this. Adding one or even two extra thicknesses of Cellophane (each approx. 30μ) does not materially affect germination, but this is retarded if spores are placed between two layers of Cellophane. Replacing the Cellophane by cellulose acetate (viscose) film does not affect germination, but has some influence on subsequent growth of the mycelium. There is no germination if Cellophane is replaced by polythene disks. If spores are placed directly on soil:agar plates without a Cellophane separation layer they germinate erratically, and if they are placed directly on a layer of damp soil they do not germinate at all. A considerable increase in the proportion of soil in the soil:agar plate decreases the germination percentage. Of the following three soil types tried: (1) a glasshouse loam, (2) an orchard soil under clean cultivation, (3) an orchard soil under grass, the third induced the most rapid germination and this is advantageous, but the three soils did not differ in the total percentage germination or in the amount of growth made by the germ tubes after 8 days. Soil after autoclaving is not stimulatory, and fails to induce any germination. Stimulation was also inhibited if 0.1 % Rose Bengal was added to the agar, but was only retarded by 200 p.p.m. streptomycin. The stimulatory properties of soil plates did not improve markedly with age but remained stationary for about 10 days and then gradually decreased. Autoclaving the soil agar plates after they had been incubated completely destroyed their stimulatory effect.

These results indicate that a substance, or substances, that induces germination of *Endogone* spores is water soluble, dialysable, produced by actively growing soil organisms and destroyed by autoclaving. Failure to induce spore germination in the cases above described can be attributed to three quite distinct and unrelated factors.

- (1) Inability of the stimulatory substances to diffuse to the spore sites, e.g. when the Cellophane becomes too dry or is replaced by polythene.
- (2) Inhibitory effects due to the fungistasis of undiluted soil, e.g. when spores are placed directly on damp soil.
- (3) Lack of stimulatory substances due to the absence of growing micro-organisms, as with autoclaved soil, or destruction of such substances, e.g. when incubated soil plates are subsequently autoclaved.

It is theoretically possible that inability to germinate, which is here attributed to the absence of micro-organisms, might be due to inhibitory substances produced in the soil by autoclaving. It will be shown later, however, that the presence of autoclaved soil particles in the medium actually improved mycelial growth of the *Endogone*. The conclusion that dialyzed metabolites of soil micro-organisms stimulate germination of *Endogone* spores is therefore thought to be the correct one. Biological stimulation of spore germination is known to occur in other fungi (Brown, 1946), but this does not usually extend to their later growth (Fries, 1943); in the *Endogone* sp., however, subsequent mycelial growth is also improved.

Upwards of 4000 resting spores have been germinated during 1958 by the use of the soil-plate method described above. Typical vesicular-arbuscular mycorrhizal infections (Pl. 16, fig. 7) have been produced by inoculation of various plants with Cellophane squares carrying sterile germinated spores.

GROWTH CHARACTERISTICS OF THE *ENDOGONE* MYCELIUM

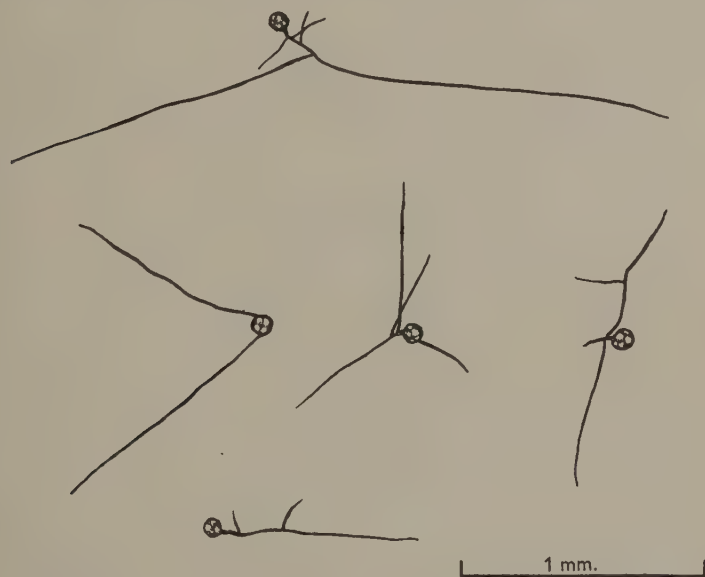
By transferring the Cellophane disks with their germinated spores to sterile soil plates made with autoclaved soil, considerable hyphal growth can be obtained. After some 200 systematic experiments, a medium has been found in which vigorous growth occurs regularly for about 10 days within a radius of *c.* 1 cm. from the edge of the Cellophane square. Thereafter growth tends to be confined to a limited number of straight, sparsely branched, questing hyphae which, within a period of 4 weeks, quite often reach the edge of the plate. It has not so far been possible to subculture from detached hyphal tips.

The mycelium produced by *Endogone* spores resembles in every particular that obtained by Butler (1939), Magrou (1946) and Stahl (1949) in previous attempts to isolate the vesicular-arbuscular endophyte. It also resembles strikingly the extra-matrical mycelium associated with mycorrhizal roots. With the method of culture described above, there is a tendency for a two-phase system to be set up, with an aerial phase on the Cellophane and a submerged phase in the surrounding soil: agar medium. Such a system appears to suit the fungus and indeed bears some similarity to its natural growth conditions, partly in the soil and partly in the plant root. It also calls to mind Dickinson's (1949) views on the growth requirements of a group of obligate parasites, the rust fungi. Growth characters of the aerial mycelium are shown in Pl. 16, fig. 6. On the Cellophane, anastomoses are extremely common, possibly because all hyphae are in the same horizontal plane and must therefore meet frequently. There is,

however, some indication that anastomoses occur not only by chance contact but that the presence of an actively growing hyphal tip near an already mature hypha will stimulate the lateral production of new peg-like processes from the mature hypha. The stimulus is not transmitted through Cellophane. One of the peg-like processes will finally join up with the approaching hyphal tip (Pl. 17, fig. 8). Butler (1939) has described the occurrence of 'angular or knob-like irregularities in the walls of main hyphae' as one of the characteristics of the vesicular-arbuscular endophyte. Anastomoses are equally common between hyphae arising from the same or from different parent spores, and growth often appears to be more vigorous afterwards. In a coenocytic fungus like the *Endogone*, such anastomoses must provide endless opportunities for the exchange of genetic material by asexual means. Another characteristic of the mycelium growing on Cellophane is the production of numerous short, thin-walled, often septate branches, illustrated in Pl. 17, fig. 9. They closely resemble Peyronel's description (1923-4) of 'lateral tufts of fine, regularly septate rhizoid-like branches', while Butler (1939) describes them as 'fine, flattened, thread-like filaments'. More recently Mosse (1954) and Nicolson (1955) have given detailed accounts of this habit of branching in the rhizosphere mycelium. Such branches are probably homologous with the structures developing into arbuscules in the plant cell. Production of absorbing branches is increased by adding acetate to the culture medium or by replacing the Cellophane with viscose (cellulose acetate) disks. Lastly the aerial mycelium is characterized by the production of spherical, thin-walled, vesicle-like spores, 15-20 μ in diameter, which are usually borne at the tip of short side branches (Pl. 17, fig. 10). They have open connexions with the hypha from which they arise, like those pictured by Stahl (1949). Occasionally hyphal growth continues from the base of the spore, in the way illustrated by Godfrey (1957) for *Endogone macrocarpa*, which shows considerable similarity to the present species in its growth habit. The new spores tend to grow into the air in an upright position at right angles to the Cellophane. The submerged mycelium is characterized by coarse, straight hyphae with sparse, almost right-angled branches and very strong apical dominance. Secondary branching is rare and this is an additional factor making this fungus difficult to culture. The strong apical dominance which is characteristic of growth in water agar:soil plates can be broken by the addition of 0.01 % Rose Bengal or by altering the balance and increasing the concentration of mineral nutrients in the medium, when a sympodial growth habit results. Branching and new spore formation also occur frequently when apical growth is temporarily stopped, e.g. when a hypha strikes a soil particle. After some delay, during which branches arise below the obstruction, the main hypha generally emerges from the opposite side of the soil particle and continues growing straight ahead. Actively growing hyphae are strongly translucent, show rapid cytoplasmic streaming and contain oil globules. At its best, their growth rate may reach 3-4 mm. in 24 hr.

GROWTH REQUIREMENTS

Hyphal growth, though quite extensive, has never been sufficiently dense to be macroscopically visible in the agar, and, since measurement by weight has not been practicable either, the comparison of different growth conditions has been based on the microscopic examination of cultures. It was assumed that the amount of growth occurring in pure water agar (typical examples of this are shown in Text-fig. 2) was due to the mobilization of spore reserves, and that any factor increasing hyphal growth beyond this was supplying some growth requirement of the fungus.

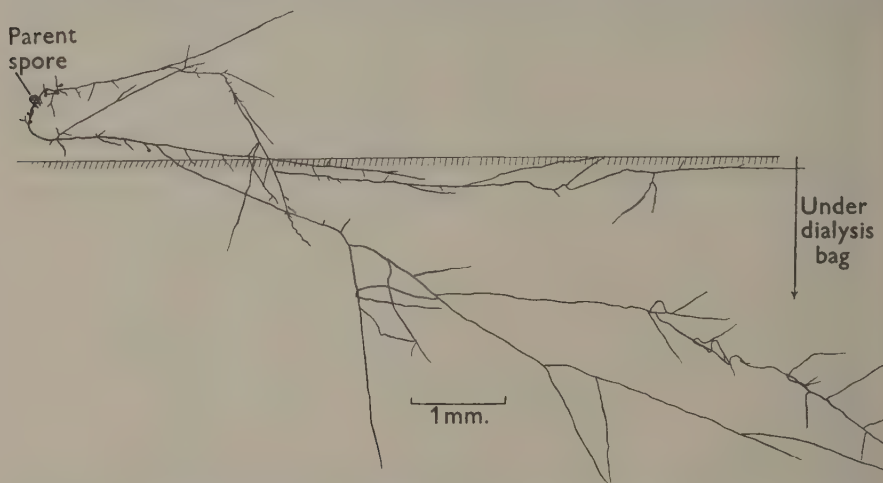


Text-fig. 2. Tracing of germinated *Endogone* spores showing character and amount of growth made by them in water agar.

Two sets of experiments based on this assumption are described below.

Series 1. Working on the commonly reached conclusion that the vesicular-arbuscular endophyte is an obligate parasite which cannot be grown in the absence of host roots, an attempt was made in 1956 to find out how far growth could be improved by root exudates, by various types of root extracts, or by growing seedlings aseptically in agar and placing germinated spores near their roots. Hyphae were not attracted to the growing roots but some growth stimulation occurred when contact was made. The stimulation was only temporary. When it was found that the parent spores were capable of initiating the same amount of hyphal growth a second and even a third time after they were severed from their original germ tubes and transferred to fresh plates, it was concluded that cessation of growth was not due to exhaustion of the spore reserves but to some inhibitory factor also present in the root or its exudates, or produced by the fungus itself. After various unsuccessful experiments with root extracts, a method

was found which occasionally did result in very marked growth improvements. If finely cut-up roots were placed in a sonic disintegrator for 5–8 min. and the resulting cell suspension was placed in dialysis bags, surrounded by water agar, and germinated spores were placed near them, then considerable hyphal growth sometimes took place, especially after hyphae had once made contact with the dialysis bags, which appeared to act somewhat like a root surface. While it was assumed at the time that the sonically disintegrated root extracts were sterile, this assumption was



Text-fig. 3. Tracing of the mycelium arising from an *Endogone* spore placed in water agar near a dialysis bag containing sonically disintegrated bean roots.

never tested, and some of them may in fact have been contaminated. It was found that disintegrated bean roots gave better results than apple or pea roots, that disintegrated shoot tissue had no effect on growth but that disintegrated *Chlorella* cultures were stimulatory and gave more consistent results than did roots. An illustration of the kind and amount of hyphal growth produced by this method is given in Text-fig. 3, which is a tracing of a culture. While these results were promising, they were never sufficiently consistent to allow a systematic investigation of the growth requirements of the fungus. In 1958 a method was found that would allow such an investigation.

Series 2. Sterile germinated spores on Cellophane squares would, when placed on sterile soil:agar plates, produce regularly, within 8 days, enough mycelium for a test of the effect of various substances on growth. Individual spores differ quite markedly in the amount of growth that they can initiate, and, rather surprisingly, this is not always proportional to their size. The use of a mixed inoculum of about twenty germinated spores therefore proved an advantage in giving more consistent results. Soil plates were made by mixing 0.3 g. fairly dry, autoclaved glasshouse loam containing a little activated charcoal with 10 ml. 0.75 % Japanese agar (equal volumes of tap and distilled water) poured just before the agar set. In pure water agar, or if the soil particles were replaced by a cold water

extract of soil, fungus growth was much less satisfactory. Soil partially sterilized by treatment with propylene oxide was equally satisfactory for mycelial development but was liable to be a source of contamination as soon as the nutrient content of the medium was raised. It is not known whether the beneficial effect of the soil particles is chemical or physical, and it is not improbable that different soils would differ in their effects, but this has not been investigated.

In order to raise the mineral nutrient level of the media, two complete culture solutions used for the growth of higher plants were tested at different concentrations. Both caused a slight improvement. The better was a solution used by Melin & Rama Das (1954) containing, per litre of distilled water, KH_2PO_4 (0.5 g.), CaCl_2 (0.05 g.), NaCl (0.025 g.), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.15 g.), KNO_3 (0.25 g.), and ferric citrate (1.2 ml of 1 % solution). This was diluted with an equal volume of tap water. No extra trace elements were added.

Having thus standardized the conditions under which a reasonable amount of growth would regularly occur, three possible growth requirements were selected for more intensive investigation; a nitrogen source, a carbon source, and the optimum pH range.

Nitrogen source. The effect of the following substances was tried: sodium nitrite, sodium nitrate, ammonium nitrate, glycine, alanine, leucine, casein hydrolysate, yeast extract, marmite and peptone. These were added to the standard medium at concentrations of approximately 0.1 %. None of these substances caused any clear improvement in growth. One difficulty was that, whereas some substances like sodium nitrate caused an initial rise in growth rate which then slowed down, others, notably casein hydrolysate and marmite, induced more steady growth after an initial lag phase, and produced about the same total amount of mycelium in the end.

Carbon source. Proceeding from the frequently expressed view that mycorrhizal infection depleted the starch reserves of invaded roots, starch and some of its hydrolysis products were tested. Starch from various sources had no effect on growth. Maltose, sucrose, and glucose were then tried at a range of concentrations and rather surprisingly had a definitely inhibitory effect. So did cellobiose though it was thought that cellulose, in the form of filter-paper, provided some substrate for the fungus. This may have been due to some physical property of the filter paper. The search was then extended to organic acids and it was found that addition of tartaric acid was definitely beneficial to growth. Oxaloacetic, pyruvic, acetic and citric acids were also promising, while malic and quinnic acids had no effect. After trying the D- and L-forms of tartaric acid and DL-tartaric acid at concentrations of 0.1, 0.2, 0.5 and 1 %, it was concluded that the best results were produced by addition of 0.2 % DL-tartaric acid and that this could be used as a carbon source by the fungus.

The strongly inhibitory effect of sugars, which had already been observed in the 1956 experiments, is interesting, and may provide a clue to the mechanism that confines vesicular-arbuscular infections to the primary cortex and thereby restricts the spread of the fungus.

Optimum pH range. It was found that the pH of the medium was one of

the most important factors controlling mycelial growth, and that, as with some other mycorrhizal fungi (Modess, 1941), optimum growth only occurred between narrow limits. The pH was adjusted by addition of N/10 NaOH to the medium before addition of the agar. It is possible that slight changes in pH occurred during autoclaving. Optimal growth was found to occur only over the range pH 6.2–6.6, and growth fell off rapidly on either side of this. This result confirms the conclusions of Magrou (1946), who found growth at pH 6.6 ten times better than at or below pH 5.2. It is also in agreement with some culture experiments of Stahl (1949), embodying an aerial and a submerged phase, where a pH of 6.7 in the submerged (liquid) phase was conducive to much better growth than one of 5.7. The marked effect of pH on mycelial development is perhaps surprising, in view of the apparent lack of correlation between soil pH and the intensity of endotrophic infection under natural conditions (Lihnell, 1939; Strzemska, 1953). It leads one to speculate whether most root surfaces could be maintained at very similar pH levels irrespective of those of the surrounding soil.

The purpose of the above experiments has been twofold; to study the growth requirements of the *Endogone* sp. and to find a medium which would serve to isolate the endophyte from naturally infected mycorrhizal roots. It is worth recording that the medium which produced maximum growth of germinated resting spores (i.e. Melin & Rama Das (1954) solution at half strength + 0.2 % tartaric acid adjusted to pH 6.5 before autoclaving) has also regularly produced some typical *Endogone* type mycelium from the cut ends and surfaces of naturally infected roots and from the broken ends of rhizosphere hyphae.

DISCUSSION

It would be gratifying to come to a conclusion regarding the probability that this fungus can be grown in culture. Very considerable growth is possible in the absence of any host root, and dialysates from biologically active soil:agar plates and from sonically disintegrated roots cause a marked growth stimulation. There is some evidence that the stimulatory substances in the soil plates are metabolites of actively growing soil organisms and it is now believed that the activity of the root dialysates may also have been due to incomplete sterility of the cell suspensions. The increase in stimulatory power with ageing of the suspensions and the generally inconsistent results obtained with them would support this view. In the culture experiments the practical difficulties of maintaining sterility have been considerable, and it has frequently been noted that a controlled amount of contamination actually led to better growth of the *Endogone* sp. All these observations suggest that any obligate relationship of this *Endogone* sp. may be with other soil organisms rather than with the host roots. The extreme lack of host specificity also supports this hypothesis.

A final conclusion could more easily be reached if the readily dialysable and therefore relatively simple stimulatory substances could be identified and if it were known whether they actually satisfy some growth requirement, or only facilitate a more rapid or more efficient utilization of the spore reserves. The inability to subculture from hyphal transplants suggests

the latter. Resting spores are so large and readily available that the extraction and incorporation of their reserves in an enrichment medium would not present insuperable difficulties. To what extent such added spore reserves could penetrate the hyphal walls is not known, but it can be demonstrated that relatively large molecules like Rose Bengal and 2:3:5-triphenyl tetrazolium chloride will accumulate in growing hyphae. The possibility is not excluded that some special physical conditions, partly fulfilled by an aerial phase on Cellophane and a submerged phase in the agar, are required by the *Endogone* sp., and account for its inability to grow from the wholly submerged hyphal transplants. At present all these possibilities are highly speculative. That they can be discussed at all in terms of controlled and repeatable experiments represents some advance.

With the information now available on sporocarp development, spore germination, and the provision of pure inocula of germinated spores, a study of the effect of this fungus on the metabolism of roots with which it has formed mycorrhizal associations becomes practicable.

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EXPLANATION OF PLATES -

PLATE 14

- Fig. 1. Sporocarps of an *Endogone* sp. associated with mycorrhizal strawberry roots.
- Fig. 2. New sporocarps developed in association with the fine rootlets of an apple seedling after soil inoculation.
- Fig. 3. Early stage in sporocarp development. No spores exist yet within the hyphal matrix.

PLATE 15

- Fig. 4. Young resting spore developing within the sporocarp matrix. Multiple hyphal attachments of the spore can be seen.
- Fig. 5. Germinated resting spore with characteristic vacuolated contents, and oil globule escaped from spore.

PLATE 16

- Fig. 6. Spores germinated on Cellophane.
- Fig. 7. Longitudinal section of an apple rootlet grown aseptically and infected from germinated *Endogone* spores on Cellophane. Arbuscules and the entry of the fungus into the root are clearly shown.

PLATE 17

- Fig. 8. Detail of anastomosing hyphae.
- Fig. 9. Short absorbing branches formed on Cellophane disk. Absorbing branches are thin-walled and, in this instance, septate.
- Fig. 10. Germinated resting spore (right) and newly formed spores in various stages of development.

(Accepted for publication 19 November 1958)

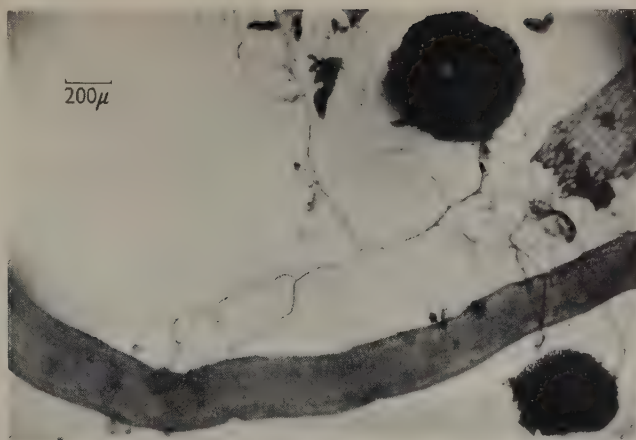


Fig. 1

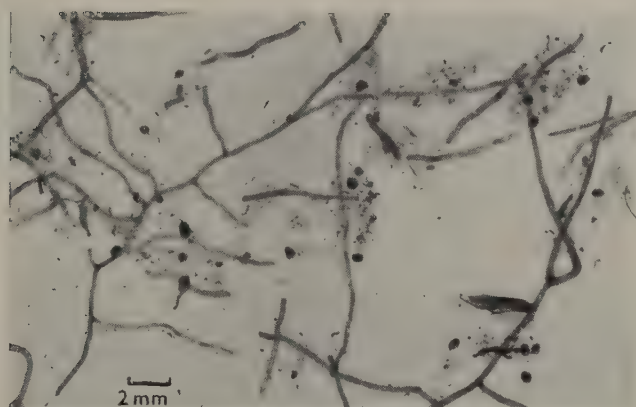


Fig. 2

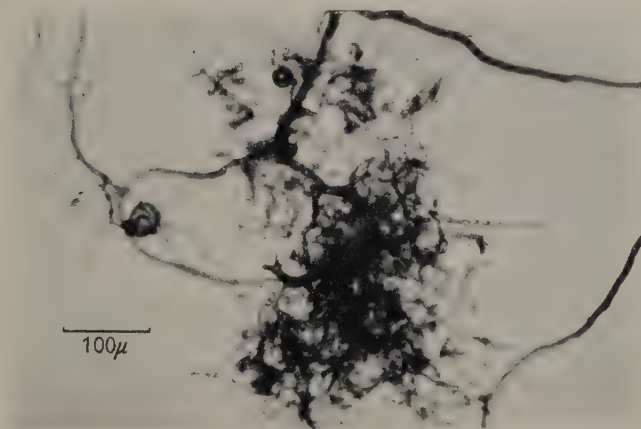


Fig. 3

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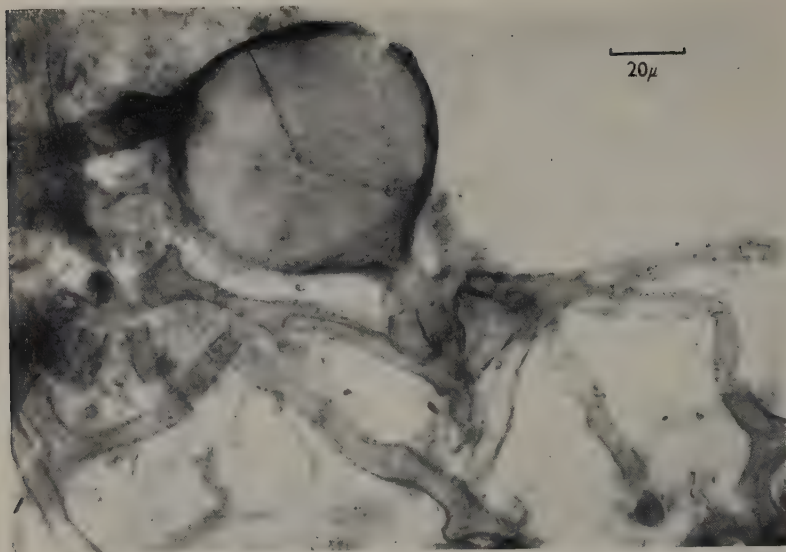


Fig. 4

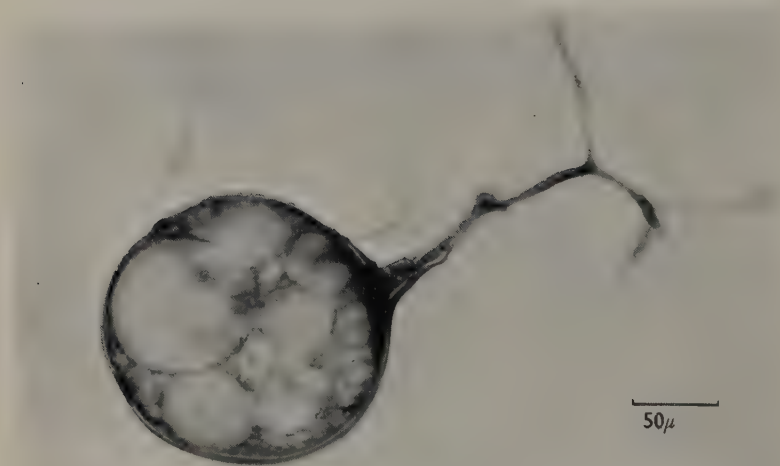


Fig. 5



Fig. 6

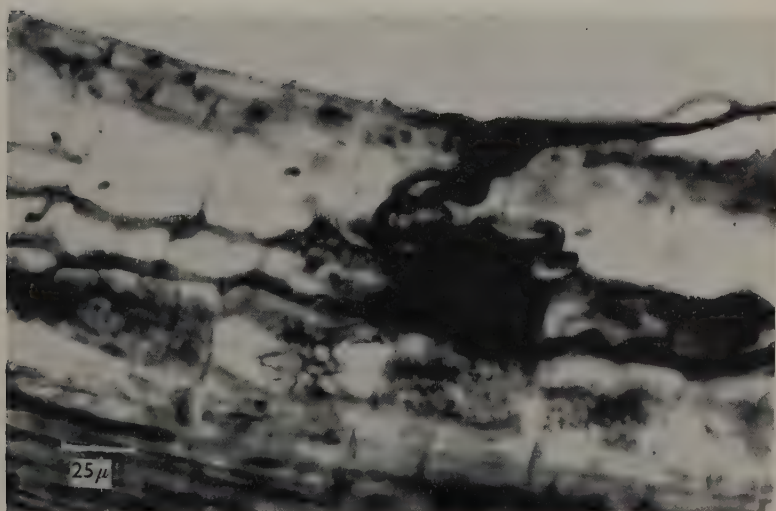


Fig. 7



Fig. 8



Fig. 9



Fig. 10

Trans. Brit. mycol. Soc. **42** (3), 287-291 (1959).

A SPECIES OF *BLASTOCLADIELLA* FROM GREAT BRITAIN

By L. G. WILLOUGHBY

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(With Plate 18 and 1 Text-figure)

A species of *Blastocladiella* is reported for the first time from Great Britain and, it is believed, from Europe. While growth in the laboratory on natural organic substrata was most prolific on keratin, it was also good on chitin, grass leaves, and hemp seeds. The zoosporangia had a pronounced stalk only when grown on hemp seeds; on all other substrata they were typically spherical with up to eight separate rhizoidal axes inserted on the lower surface, and up to three exit tubes on the upper surface. Pure cultures were made on YpSs agar, and resting spores were obtained from a single spore isolate grown on glucose-peptone-yeast extract agar. Indications so far are that the taxonomic position is close to *B. simplex* Matthews and *B. laevisperma* Couch & Whiffen, but the living zoospores appear to be larger than those of most described species of *Blastocladiella*, and comparable in this respect with those of *B. emersonii* Cantino & Hyatt and of *Allomyces* species.

The fungus was obtained during the course of routine sampling from Esthwaite Water (Lake District) where an ecological investigation on the lower saprophytic fungi is being carried out. The baiting methods outlined in previous publications (Willoughby, 1956, 1957) are being used on mud and soil samples collected at three levels on a line transect at a single site. The levels are (i) the Littorella zone at 2 m. depth, (ii) the marginal zone where the soil layer is a thin one overlying gravel, and which is periodically inundated when lake levels are high, and (iii) the adjacent pasture field. Sampling is carried out at approximately fortnightly intervals.

So far the *Blastocladiella* species has been recovered only from samples collected from level (ii). The resting sporangia were observed first (on grass-leaf baits), but no rhizoids were seen. More thalli were obtained on grass leaves and other baits from further samples, and eventually particularly clear material growing on snake-skin bait clearly indicated that a species of *Blastocladiella* was present. In this material there were both resting sporangia and zoosporangia, and the latter readily produced zoospores of the *Allomyces* type, with a well-marked nuclear cap. Rough cultures were made by looping zoospores into flasks containing sterile snake skin and lake water, and these were carried on for several weeks; but a species of *Chytriumyces* which was also present grew more prolifically than the *Blastocladiella* and completely replaced it in several flasks. The method adopted to obtain pure single spore isolates from these crude laboratory cultures was as follows. Two mature zoosporangia with a small

portion of the substratum were dissected out into a drop of penicillin-streptomycin mixture on poured plates of 2% quarter-strength YpSs (yeast, phosphate, soluble starch) agar. Dehiscence occurred within a few minutes in both, apparently induced by this manipulation. Zoospores



Text-fig. 1. A species of *Blastocladiella*. a, zoospores; b, zoospores germinating on agar; c, a young thallus after 24 hr. growth on agar; d, a young thallus showing two rhizoidal axes; e, a mature zoosporangium; f, a mature zoosporangium grown on hemp seed illustrating the 'stalked' type of thallus; zoospore outlines are visible in the upper cell only; g, a large exit pore; h-j, resting sporangia. The resting spore is drawn in optical section in h and j, and in surface view in i, where numerous minute pits are visible. a is freehand, but based on measurements made on living material; the remainder are camera-lucida drawings. All other drawings are of dehiscid sporangia, illustrating the range of form observed; b, d, and e at scale below d, others, except a, at scale to right of f.

emerged individually, although the exit tube was wide enough to permit several to escape simultaneously, and complete evacuation took approximately 30 min. When zoospores were emerging, those inside swarmed vigorously. It was therefore possible to remove the zoosporangium to another agar plate after a number of zoospores had been released, and this procedure continued until dehiscence was over and a number of plates had been inoculated. The antibiotic drops were run over the surface of the plates, which were incubated at 20° C. After 24 hr. abundant germination had occurred (Text-fig. 1c), and a number of individual thalli were cut out with sterile needles and transferred to slopes of YpSs agar. Others were placed in sterilized flasks of lake water plus snake skin. In this way a number of pure single spore isolates was obtained. One was studied intensively, and the following data obtained from it. Zoospores (Text-fig. 1a) were hyaline and oval when in motion, 7.5–11 μ long by 4.5–6 μ wide, and 7.5–9.5 μ diam. when at rest in a spherical shape. The posterior flagellum was 25–27 μ long. Detailed internal structure agreed closely with published accounts of *Blastocladiella* and *Allomyces*. Zoosporangia on glucose-peptone-yeast extract agar (gpya) were spherical, 35–196 μ diam., with up to three exit tubes. On gpya and YpSs with 1.5 and 2 % agar it was commonly observed that, although zoosporangia readily reached their mature size, the formation of exit tubes and release of zoospores was largely dependent on the addition of water at this stage. When water was added, exit tubes at once began to bulge out, and mass dehiscence was in progress within a few hours (Pl. 18a, b). Pure subcultures were established on termite wings (chitin), snake skin (keratin), grass leaves, and hemp seed suspended in lake water. Growth was good on all these substrata, but most prolific on keratin. There was no growth on cellophane. Sporangia were 9–110 μ diam. on chitin (Pl. 18c), 10–140 μ on keratin, and 22–128 μ on grass leaves. On keratin, oval sporangia were not unusual, and these were 24 $\times$ 30 μ to 80 $\times$ 127 μ . In hemp seed subcultures most thalli were distinctly clavate in shape, up to 200 μ long and 105 μ wide. The zoosporangium was cut off by a cross septum just below the widest point, and thus on this substratum only the 'stalked' thallus was typical (Text-fig. 1f). Couch & Whiffen (1942) have reported a similar state of affairs in *B. laevisperma*. In the single spore isolate studied, resting sporangia were obtained on chitin, grass leaves, hemp seed and agar, and were 42–94 μ diam. An attempt was made to induce resting sporangium formation on agar following the procedure of Cantino (1956). With concentrations of 0.5×10^{-2} M and higher of NaHCO₃ incorporated in gpya, growth was inhibited completely. With 0.05×10^{-2} M-NaHCO₃ (a concentration well below the critical one for *B. emersonii*), although the first generation derived from zoospores was 100 % zoosporangial thalli, a number of resting sporangia were found in successive generations which developed as dehiscence proceeded on the dilute agar used (1.5 %) and dense pustules of thalli built up slowly. So far resting sporangia have not been found on YpSs agar.

In all the material studied, including the single spore isolate, the crude subcultures, and thalli from the initial soil baitings, exit tubes were never more than three in number, and up to 123 μ in length. They were 4–24 μ

wide at the base and $4-15\mu$ at the pore (Text-fig. 1). Rhizoids were up to 22μ wide. Resting spores (Text-fig. 1*h-j*, Pl. 1*d, e*) were spherical, $25-95\mu$ diam., and never completely filled the parent thallus, which was also always spherical and $30-115\mu$ in diameter. Resting sporangia had one to eight separate rhizoidal axes; a deep reddish brown pigmentation and minute pitting on the outer wall were constant features when mature.

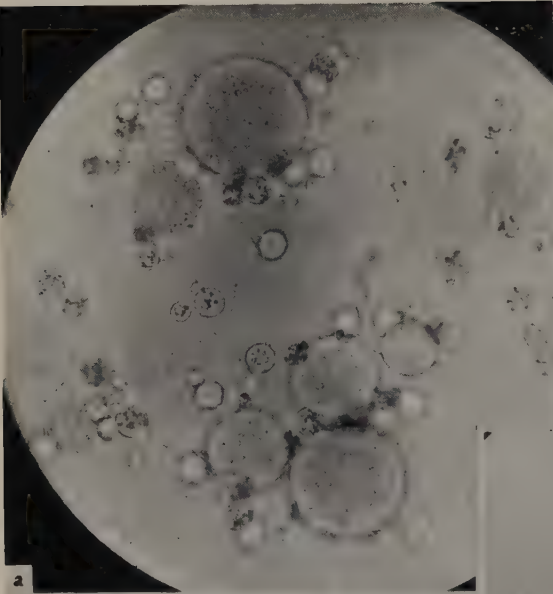
Since the resting spores have not yet been germinated, and the complete life-cycle is not known, a final specific determination is not possible. However, morphological characters which may prove useful in specific delimitation relate to the zoosporangia and the zoospores. On all substrata, including agar but with the exception of hemp seeds, the zoosporangia are typically spherical with up to eight separate rhizoidal axes inserted on a small area on the lower surface and one to three exit tubes on the upper surface. The zoospores, which appear to be larger than most described species of *Blastocladiella*, are comparable in this respect with those of *B. emersonii* (Cantino & Hyatt, 1953) and of *Allomyces* species. However, it would be unwise to attach too great an importance to the latter character, since measurements on living zoospores are particularly susceptible to error. If, as anticipated, the life-cycle should prove to be of the 'brachy' type, then the Esthwaite fungus is clearly very close to *B. simplex* (Matthews, 1937) and *B. laevisperma* Couch & Whiffen, but differs from the latter in that the resting spore wall never appears to be ridged. In *B. emersonii*, isolated thalli always form well-defined short stalks, and thus my fungus cannot be placed in this species.

The main reason for writing this account is that a *Blastocladiella* species has never before been reported from Great Britain, or even from Europe as far as can be ascertained. All previous records are from the American continent. It is of interest to record that *Blastocladia pringsheimii* Reinsch, a common species in Great Britain, and well known through the researches of Blackwell (1940), Lloyd (1938), and Waterhouse (1942), has also been found in Esthwaite Water on a number of occasions when the appropriate trapping methods have been used.

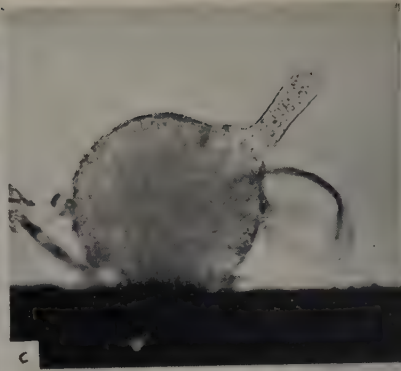
My thanks are due to Prof. C. T. Ingold for kindly reading and commenting on the manuscript.

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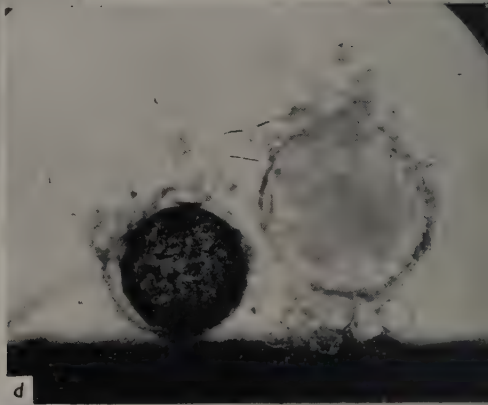
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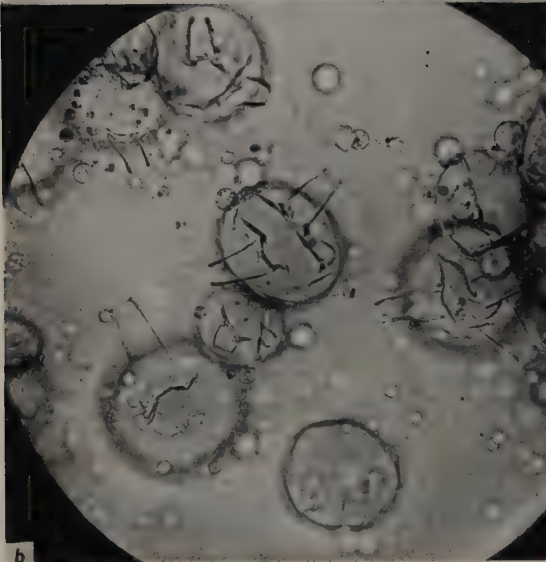
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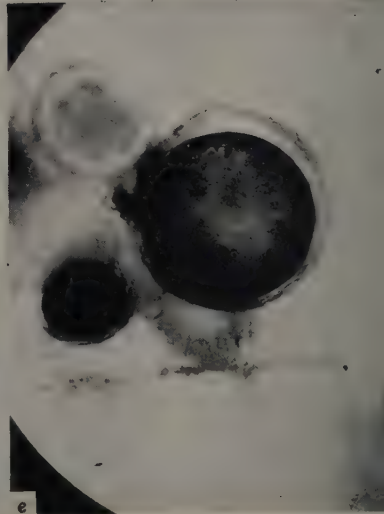
c



d



b



e

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EXPLANATION OF PLATE 18

A species of *Blastocладиella*

a, mature zoosporangia on glucose-peptone-yeast extract agar. Many young thalli are also present. *b*, a field from the same Petri dish as *a*, but after flooding with water. Dehiscence has occurred in all mature sporangia, and exit tubes are now obvious. *c*, a mature zoosporangium on termite wing substratum. *d*, *e*, resting sporangia on termite wing substratum. Photographs ($\times 280$) are all of material derived from a single spore isolate.

(Accepted for publication 3 October 1958)

STUDIES ON SOIL CHYTRIDS

IV. A CHYTRID PARASITE OF ROTIFER EGGS

By L. G. WILLOUGHBY

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(With one Text-figure)

A chytrid very similar to *Rhizophyidium zoophthorum* (Dangeard) Fischer is described. It occurred as a parasite on the eggs of the rotifer *Lecane* (*Monostyla*) *arcuata* Bryce.

The chytrid was found on a single occasion, parasitic on the eggs of the rotifer *Lecane* (*Monostyla*) *arcuata* Bryce, when soil from a pasture field adjoining Esthwaite Water (Lake District) was baited with grass leaves, primarily in order to obtain lower saprophytic fungi.

In the material examined, one rotifer species was present, as far as could be ascertained, and the adults browsed actively on the grass leaves. It was possible to follow a complete developmental sequence of the uninfected host from egg to adult; and it was clear that only the very youngest egg stage was attacked by the fungus. The uninfected egg consisted of a central sac of dense material surrounded by a distinct wall, suspended in a clear zone of material which was itself enclosed by a further outer wall. The rotifer developed in, and eventually emerged from the central sac. Mr A. L. Galliford, who kindly examined some of my material and identified the adult rotifer, points out that parthenogenetic female rotifer eggs (which mine evidently were) normally consist of a dense egg-shaped or spherical body only, and lack the outer clear zone and wall. However, I see no alternative but to rely on my own observations in this respect.

The zoospore of the chytrid was $3-4\ \mu$ diam., with a hyaline globule and nuclear cap (Fig. 1*a*). It settled down on the outer wall of the egg (Fig. 1*b, c*), and rhizoids were clearly visible, even in the youngest stages, traversing the clear zone (Fig. 1*d-f*) on their way to the central dense material. Some of the rhizoids were extremely robust and extended up to $37\ \mu$ (Fig. 1*e-g*). In eggs which were presumably in a late stage of attack (Fig. 1*g-h*), it was impossible to distinguish the membrane of the inner dense material of the egg, and eventually (Fig. 1*i*) the latter had no clearly organized contents. Mature sporangia of the chytrid were spherical, $7.5-26\ \mu$ diam., and dehiscence was inoperculate, the zoospores escaping from the apex which projected very slightly in this region (Fig. 1*g, i*). Although resting spores were not observed, the morphology and dehiscence indicated that the chytrid was a *Rhizophyidium*. The eggs of the rotifer appeared viable, and hence it is suggested that the chytrid was parasitic. In a study of the saprophytic chytrids present at the site

from which the soil sample was collected, no species has so far come to light to which the *Rhizophydium* could confidently be assigned.

The Esthwaite fungus is clearly very similar to *R. zoophthorum* (Dangeard) Fischer, a parasite of rotifer eggs, and recently re-described by Karling (1946). However, my specimens lacked the prominent tapering apex which Karling states is characteristic of this species.

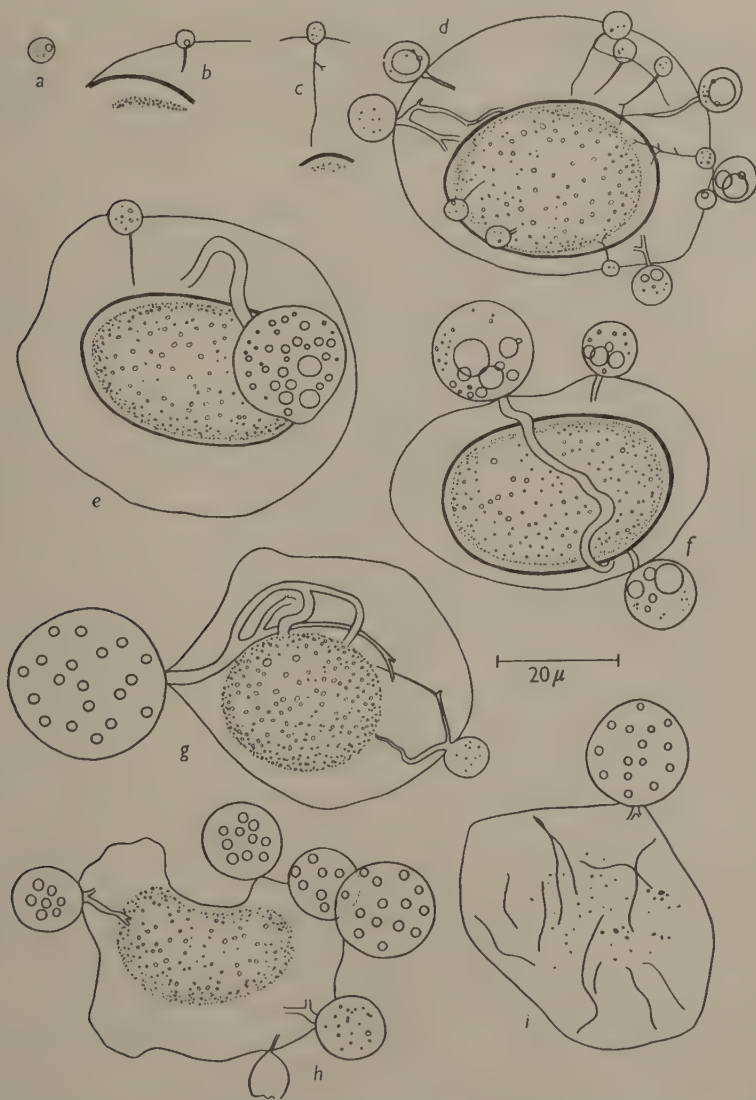


Fig. 1. *a*, zoospore; *b*, *c*, young stages on the host; *d-f*, parasitized eggs with many young stages of the chytrid; *g*, *h*, parasitized eggs with mature sporangia of the chytrid (the limiting membrane of the inner egg sac is no longer clearly visible); *i*, a late stage of infection with a single mature chytrid sporangium visible (there are no longer any organized contents in the egg). All drawings to the same scale.

My thanks are due to Mr A. L. Galliford (a member of our Association) who provided measurements and illustrations of the rotifer.

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(Accepted for publication 3 October 1958)

SPORANGIAL WALL PATTERNS IN *PILOBOLUS CRYSTALLINUS* TODE

By VIVIANNE J. DRING

(With 1 Text-figure)

The observations of earlier workers on sporangial wall patterns have been confirmed for some strains of *Pilobolus crystallinus*.

Patterns on sporangial walls of *Pilobolus crystallinus* have been observed by Coemans (1861), van Tieghem (1876) and Zopf (1888, plate XXII; 1890, fig. 54), who all described or pictured these patterns as dark hexagons separated by a network of paler or white lines, there being a central hexagon at the apex of the sporangium surrounded by six lateral ones.

These observations were only partly accepted by Buller (1934) who, failing to find similar reticulations in three other species (*P. kleinii*, *P. longipes* and *P. umbonatus*), came to the conclusion that the earlier authors had been describing 'the wrinkling of the hemispherical sporangial wall as this settles down on the drying mass of spores. . . . The white lines seen by Coemans and van Tieghem were doubtless ridges around depressions.' He was also of the opinion that Zopf had been in error in representing a pattern on the undischarged sporangia of *P. crystallinus*.

While investigating the occurrence of species of *Pilobolus* in Ghana an isolate of *P. crystallinus* was obtained from hutch-rabbit dung from Bunso, Southern Ghana, which showed clearly the reticulations previously described for this species. Microscopic examination with bright illumination of discharged or detached sporangia revealed that the hexagons, separated from each other by a region of colourless material 4-6 μ wide (Fig. 1*a*), are not uniformly black but have irregularly darkened areas at their centres (Fig. 1*b*) (Coemans, 1861). If pressure is applied from above to a sporangium, it tends to split along the colourless lines (Fig. 1*c*), usually not in the middle of a colourless band but along one edge of it. The sporangia are not all exactly similar in their patterns, five- and seven-sided forms being quite common (Fig. 1*b*).

After the isolate had been kept in culture for several months, the patterns became very irregular (Fig. 1*d, e*) and indistinct. Zopf was, in fact, right in depicting patterned sporangia seated on their sporangiophores, as the pattern first appears while the sporangium is still immature, orange and rounded (Fig. 1*f*). As it matures the sporangium becomes faceted because the hexagons become flattened and rigid (Fig. 1*g*).

Altogether six different isolates of *P. crystallinus* were obtained from three different kinds of dung (goat, horse and rabbit) from three different localities (Aburi, Accra and Bunso). Two showed the patterns clearly and

regularly and four showed no sign of them. This agrees largely with Coemans's observations, though he regarded the presence or absence of patterns to be concerned with heat- and light-factors. Dried discharged sporangia from an unpatterned isolate were compared with similar

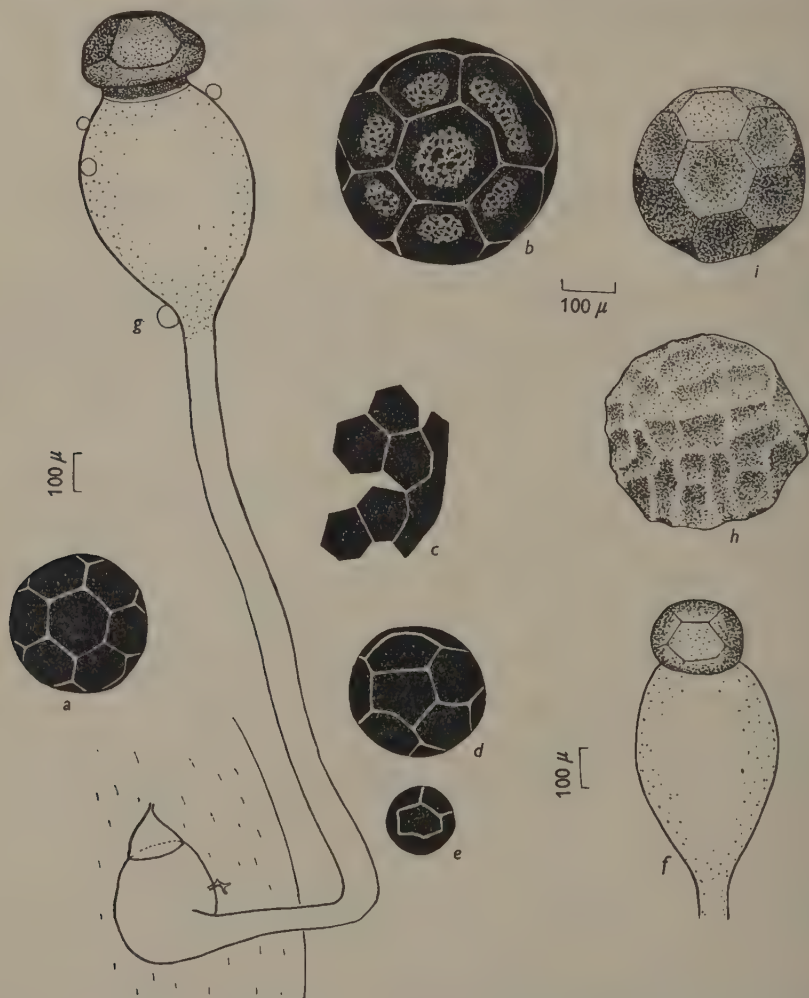


Fig. 1. *Pilobolus crystallinus*. *a*, discharged sporangium showing hexagonal pattern; *b*, discharged sporangium showing details of wall structure; *c*, part of discharged sporangium to which pressure has been applied; *d*, *e*, sporangia from 6-month-old culture; *f*, immature sporangium; *g*, mature fruit-body; *h*, dried discharged sporangium of an unpatterned isolate; *i*, dried discharged sporangium of a patterned isolate.

sporangia from a patterned isolate (Fig. 1 *h*, *i*). The unpatterned ones wrinkled in a manner very similar to those of *P. longipes* as illustrated by Buller (1934), while the patterned ones retained their faceted appearance, each facet becoming very slightly depressed at the centre. It seems clear, therefore, that Buller's conclusions could apply only to unpatterned strains

of *P. crystallinus*, and the observations of Coemans, van Tieghem and Zopf remain valid.

Cultures and slides of *P. crystallinus* have been deposited at the Commonwealth Mycological Institute, Kew.

I wish to express my thanks to Prof. C. T. Ingold for his kind help.

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(Accepted for publication 17 October 1958)

Trans. Brit. mycol. Soc. **42** (3), 298-307 (1959).

THE CHOKE DISEASE OF COCKSFOOT (*DACTYLIS GLOMERATA*) CAUSED BY *EPICHLÖE TYPHINA* (FR.) TUL.

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(With Plate 19 and 1 Text-figure)

Investigations to ascertain the means by which infection of *Dactylis glomerata* by *Epichloe typhina* is accomplished are described. The results of experiments on seed transmission by spore contamination or by flower infection suggest that neither occurs and field data from a survey of crops of known origin support this view. Spore trapping and other observations have shown that the peak formation of conidia occurs rather before maximum flowering (anthesis) and ascospores are not released in quantity until flowering is virtually over. Ascospores and conidia have been shown to germinate on the cut surfaces of cocksfoot stubble and the passage of hyphae down the pith has been traced. Experimental inoculations of stubble ends have resulted in stromata the following season.

The choke disease of grasses is no newcomer to British agriculture. The pathogen, *Epichloe typhina* (Pers. ex Fr.) Tul., has long been indigenous here and over twenty grass hosts are known, though only a minority are of any economic importance. The most comprehensive account of the disease in Britain is by Sampson (1933) who traced the life history of the fungus on *Festuca rubra* L. Miss Sampson showed conclusively that in this grass *Epichloe* is transmitted by seed, infection of which is brought about by hyphae which grow up through the flowering stem from the base of the plant and penetrate the developing ovary. Such seeds are viable or not according to the degree of damage to the tissues and to the extent of the mycelium within them. The fact that commercial seed of red fescue does contain the fungus has been confirmed by observations made in routine seed testing (e.g. Wernham, 1942).

By far the most important agricultural grass attacked in Britain by *E. typhina* is cocksfoot but here the host-parasite relationship is unlike that in *F. rubra*. The appearance of the stromatic sheath is quite distinct on the two hosts. In the fescue the inflorescence is not completely suppressed, and viable, though frequently infected, seeds are produced on stems obviously heavily parasitized. This is not so with cocksfoot on which the stromatic sheath is so complete that, with few exceptions, total suppression of the panicle occurs. The means by which *Dactylis glomerata* becomes infected in the field have never been established and the present paper deals with experimental evidence on this obviously important matter.

THE COCKSFOOT DISEASE

As a result of observation and experiment extending over many years certain peculiarities of the disease on cocksfoot have become known and may be summarized as follows.

(1) An infected plant usually produces infected heads every year and the proportion of these tends to increase in succeeding years.

(2) With rare exceptions, crops in the year following seed sowing (i.e. in their first harvest year) show no sign of the disease. In the second harvest year a little choke often occurs and in the third and later years heavy infections (up to an extreme case of 80 % of the potentially fertile tillers) are common.

(3) Choke usually appears first as a light infection well scattered throughout the crop.

(4) Choked panicles of cocksfoot always have abundant mycelium in the pith of the stem; healthy panicles on infected plants never do.

(5) The incidence of the disease does not appear to be influenced to any recognizable extent by soil type, fertility or lime content.

(6) *Epichloe* attacks all types of cocksfoot and may be found in old pastures in all parts of the country, though, as a result of grazing and the small size of the plants, its presence may be overlooked.

(7) There appears to be no spread of disease from infected to healthy individuals when grown as spaced plants in blocks.

The fungus

Two fruiting stages of *E. typhina* occur regularly on *D. glomerata*. In May hyphae emerge from within the host tissues and form a stroma which increases in thickness and completely smothers the developing inflorescence. The hyphae and the stroma are white at first and minute single-celled oval conidia are produced in profusion. These can produce germ tubes when mature. They are dispersed by wind but much more easily when this contains droplets of water. Towards the end of June (in the north) the conidia are gradually superseded by perithecia and the stroma colour changes from white to orange. The perithecia are embedded in the stroma and produce numerous asci each containing eight filiform, septate ascospores capable of immediate germination following their extrusion through the ostiole. Spore discharge continues over a considerable period and is in full activity at the time the crop is harvested in July.

The role of the conidia and ascospores in the life history of *E. typhina* on cocksfoot was unknown, sustained attempts to induce infection by a variety of methods with both having consistently failed. The present paper deals with further experiments designed to establish the possibility of infection through (a) the seed or (b) the vegetative parts of the host.

SEED TRANSMISSION

Field observations on the incidence of *E. typhina* in cocksfoot, especially the apparently random nature of its first appearance, suggest that some form of seed transmission is involved. If this is so the almost invariable

absence of stromata from crops in their first harvest year is puzzling. This well-established observation was recently confirmed by the results of the survey reported by Large (1954). It is known (Sampson, 1933) that infected plants sometimes show no external evidence of their condition and the existence of this 'latent infection' on a wide scale might possibly explain the absence of first year symptoms. There is no evidence, however, that this does occur on such a scale and it is difficult to visualize the way in which it might be brought about. Infection of the seed could conceivably occur in three ways: (i) by hyphae growing up the flowering stem as in *F. rubra*, (ii) by spore contamination of the seed, leading to seedling infection, and (iii) by a form of flower infection similar to that known in the smut fungi.

The first possibility has been investigated by examining, in quantity, pith scrapings of flowering stems but in none have we found hyphae in those producing normal panicles. Flower infection might appear a likely possibility, and Diehl (1950) in his work on the related fungi, the *Balansiae*, successfully inoculated stigmas of *Cenchrus echinatus* L. with conidia of *Balansia obtecta*. The plus sign in his table, indicating that stigma infection has been proved for *E. typhina*, is an error and should be disregarded. The parasitism of *Epichloe* is similar in many respects to that of the smuts and in this group flower infection occurs with considerable frequency. The possibility of seed contamination or of flower infection as a means of transmission was tested as follows.

Seed contamination

(1) Seed was threshed from sheaves to which a considerable amount of perithecial material had been added. No viable ascospores were recovered from this seed and no *Epichloe* mycelium was observed in representative seedlings. Drills were planted in the field but no stromata developed at any time during the next 3 years.

(2) Seed was harvested from a very heavily infected field. Washings from the dry and mature seeds contained ascospores but these were dead. Seeds from this source were also microtomed and a large number of embryos examined by the method described by Simmonds (1946), but no invasion of host tissues by *Epichloe* was found. Drills of check plants kept in the field for 3 years remained healthy.

(3) Inoculations of growing inflorescences were carried out using a modification of the vacuum pump apparatus described by Moore (1936). Heads of all ages from pre-flowering to seeding were inoculated with conidia and ascospores as these became available. Representative samples of all inoculations were examined microscopically and enough seed saved for drilling in the field. No infection was observed in the laboratory and no stromata developed in the field in the 3 years following treatment.

(4) Conidia and ascospores were applied directly to the stigmas by various methods. Diehl (1950) made many such inoculations on several hosts of species of *Balansia*; most were unsuccessful but positive results were obtained with *Cenchrus echinatus* when conidia of *B. obtecta* were placed dry on receptive stigmas. Twenty plants were raised from this material of

which three were infected. Spraying a suspension of conidia on to similar stigmas Diehl found completely ineffective.

In Leeds several methods were tried. Some flowers were emasculated and bagged, inoculated with conidia when the stigmas were exerted and pollinated the next day. Others were pollinated and later inoculated and some were dusted with conidia on a dry brush before the stigmas were exerted. Suspensions of conidia were also used and humidity tubes, containing saturated cotton wool, were placed over the inoculated heads to prevent premature drying out of the stigmas. Samples of all inoculations were examined under the microscope to trace the development or fate of the conidia deposited on the stigmas.

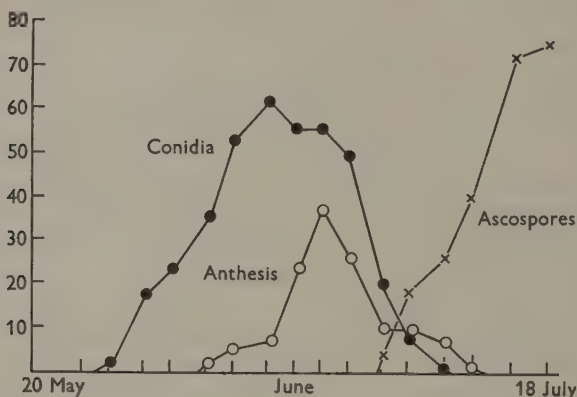
The humidity tubes greatly increased the chances of survival of the deposited conidia and within them germination was good and hyphae grew freely on the surface of the stigmas but no case of actual penetration was observed. Fixed and stained preparations showed that hyphae grew as far as the base of the stigma but they remained entirely superficial and no entry into the ovule itself took place (Pl. 19, fig. 3). Seeds from these experiments were drilled in the field but no *Epichloe* developed in the three succeeding harvest years.

(5) The possibility of seedling infection was investigated by attempting to induce penetration following germination of the seed. Seeds were germinated on active cultures of *Epichloe*, and various other methods were devised to bring inoculum into close contact with the young coleoptiles and other parts of the germinating embryo. No plant from these experiments produced stromata in the field during the following 3 years, but a microscopical examination of inoculated seedlings showed that a vigorous growth of hyphae occurred over the basal parts of the young shoot and also on the root, but again no actual invasion of the host tissues was observed. A constant feature of these inoculated seedlings was the presence of a mass of mycelium lodged in the cleft between the base of the plumule and the scutellum. This mycelium was frequently disposed in a definite pattern with an orderly arrangement of hyphae with their tips in close association with the plumule cells. No penetration of these cells occurred and observations continued for 6 days showed no further development of the hyphae. These results, and others from similar experiments, suggest that the seedling is unlikely to be the source of initial infection.

SPORE LIBERATION AND TRAPPING

The discharge of ascospores by *E. typhina* has been described by Ingold (1948) who showed that, in the laboratory, the process continued for several days provided that the host tissue was adequately supplied with water. He noted a periodicity in spore liberation with a morning minimum increasing to a maximum in the late afternoon or evening. The release of conidia was studied by us and experiment showed that many more of these spores could be recovered on trap slides when stromata were subjected to a current of air carrying atomized water than when a similar current of dry air was used. A humid air stream also favours survival since conidia shrivel very rapidly on drying and it is probable that many of those

produced in nature are destroyed under conditions of low humidity. Spore trapping was carried out in a heavily and uniformly infected field near York using a modification of the apparatus described by Hyde & Williams (1945). The traps were mounted on wooden dowels which fitted into lengths of gas piping driven into the ground. The height could be adjusted at will and the trap turned in any required direction. Data were collected from seven positions in the field and the slides proved efficient in the collection of cocksfoot pollen, conidia and ascospores.



Text-fig. 1. The production of conidia and ascospores in relation to the flowering period of cocksfoot. Percentages of tillers in each stage at the dates shown.

The counts of conidia were the most difficult to make owing to their small size and similarity to other bodies trapped on the slide. The field trap data were, however, completely confirmed by the results of observations on the maturation of stromata and healthy panicles, which extended from the first appearance of *Epichloe* and the emergence of the flowering head up to harvest when ascospore production was at its peak. To do this six stages in the development of the stroma from the earliest conidial to the completely perithecial stage were defined and the percentage of individuals in each category at the various sampling dates was determined. A comparable classification was devised for the development of the inflorescence from before anthesis to seed maturation and similar counts were made. The results, which are in agreement with those from the trap counts, are shown in Text-fig. 1. It would appear from these that if stigma infection occurs at all it must be by means of the conidia and not by the ascospores since these are not produced in any number until well after the pollination period. In any field of cocksfoot, however, there are panicles that are much later than the average and it is conceivable that these might be infected by ascospores. There is no evidence that this happens and it seems most unlikely that these few chance ascospores could produce the widespread stromata that are such a feature of the disease.

VIABILITY OF ASCOSPORES

For three seasons collections of perithecial stromata were stored under various conditions under partial cover and in the open air with a view to determining their longevity. The course of events in all three years was the same, namely a very high germination capacity in July followed by a steady diminution of viability until November when only 14 % of the stromata yielded any viable spores at all and these were few. Isolated spores were found to germinate up to the end of January, then there was no further sign of life. In the field *Epichloe* stromata appear to be palatable to insects and other small animals as well as being heavily overgrown by moulds, such as *Cladosporium* and *Penicillium*. It seems most unlikely therefore that any appreciable number of ascospores would survive the winter and act as a source of infection the following season.

SEED TREATMENT

The possibility of seed transmission was further tested by the use of an organo-mercury fungicide and warm water disinfection on seed harvested from a very heavily infected (40–60 %) crop of S. 143. Drills of the various treatments and controls were sown in Leeds, Aberystwyth and Newcastle and kept under observation for 3 years. No *Epichloe* developed in any treatment or in the controls.

The results of the experiments so far considered suggest that choke in cocksfoot is not carried by the seed, although this is produced in such quantity in the field that a very small percentage of infection might be sufficient to introduce the disease to fresh crops. If this is so, one would expect to find more *Epichloe* in stands grown from seed obtained from crops in their second harvest year, or later, than from seed of first year crops which are virtually free from stromata. Through the kindness of the Director of the Welsh Plant Breeding Station and Mr T. E. Miln of Messrs Gartons, Warrington, a list of fields was compiled and a survey made of them. The results are shown in Table 1. The figures show that choke occurs regularly

Table 1. *Incidence of Epichloe in third year crops from seed harvested in the 1st and 2nd years of the parent crops*

Source of seed	Incidence of <i>Epichloe</i>	
	Present	Absent
1st year crops	19	30
2nd year crops	34	60

in stands of cocksfoot sown with seed from 1st year, and presumably *Epichloe* free, crops. The proportion of infected to clean crops is about the same as with the 2nd year seed and is essentially similar to comparable figures for seed obtained from crops of all ages (i.e. 1st, 2nd, 3rd and 4th harvest years).

The information from this source supports the conclusion drawn from the inoculation studies that seed transmission is not an important source of field infection.

THE SIGNIFICANCE OF PARTIALLY EMERGED HEADS IN COCKSFOOT

As a general rule the stromata of *E. typhina* completely suppress the inflorescence of *D. glomerata*, but occasionally a crop is found containing a proportion of partially emerged heads (Pl. 19, fig. 1). This condition, in our experience, is most likely to be found in crops in their second year and we have not found it in those older than this. One field in Wales was of particular interest in that it had been sown in two parts, the first in the autumn and the second in the following spring. No *Epichloe* developed in the first harvest year, but it was abundant in the year following and partially emerged heads were found in profusion. The distribution of these was of interest in that there were twice as many in the spring sown (and younger) block, even though the total *Epichloe* (i.e. fully and partially suppressed panicles) was greater in the older portion. This suggests that the fungus needs time in the host to grow sufficiently to invest the panicles completely. Initial infection must therefore have occurred a considerable time before the appearance of stromata and two possibilities become apparent. Either the seed is infected and 2 years are needed for the expression of symptoms, or infection occurs sometime during the first growing season. An opportunity occurred in 1953 to examine the first of these alternatives, when partially emerged heads were collected from a field where they were present in quantity. Examination under the microscope showed that many seeds were invaded by *Epichloe* which had stopped the development of some at an early stage (Pl. 19, fig. 2). All stages were found from complete abortion to apparently sound seeds that germinated normally. It was possible to grow on 116 of these seedlings and the resulting plants were carefully searched for signs of infection in each of the three following harvest years but with negative results. It seems therefore that although infected seeds may well be harvested from these partially emerged heads they are non-viable and field transmission of the disease by this means is improbable.

TRANSMISSION THROUGH INFECTION OF THE VEGETATIVE PARTS OF THE HOST

The prolific production of spores by *E. typhina* at a time when the host plant is in full vegetative vigour suggests that some form of penetration of leaves or sheaths might occur. No visible signs of such penetration have been reported on cocksfoot plants and no evidence has been recorded of penetration of vegetative tissues other than the work of Vladimirskaja (1928) who claimed successful inoculation of tiller buds. She did not say that this resulted in the production of stromata, and Sampson (1933) has pointed out that since apparently healthy plants sometimes carry *Epichloe* mycelium it is possible that Vladimirskaja was working with plants of this type. Using large numbers of plants, we have attempted to obtain infection of plants in the vegetative condition in the ways summarized below.

Methods used in inoculating vegetative plants

(1) Successional sowings were made of cocksfoot seed to give plants in all vegetative growth stages. They were kept under conditions of high

humidity and sprayed at 3-day intervals from June onwards with suspensions of conidia followed by ascospores when available.

(2) Seedlings and plants in various vegetative stages were grown in pots and stromata (conidial and ascigerous) in tubes of water were inserted in each. High humidity was maintained and spore discharge was heavy. The plants were kept under treatment for 6 days.

(3) Pots of young plants were arranged round heavily infected plants growing in the field. Both conidial and perithecial stages were used.

(4) Tiller buds were inoculated with conidia, ascospores, and mycelium from pure cultures; both surface and hypodermic inoculations were made.

Plants from all the above experiments were grown on in the field for 3 years but in none were stromata produced. These experiments confirm the results of others made previously by the senior author and are also in accordance with direct observations of the behaviour of individual hyphae and germ tubes on the epidermis of the host. These often grow well for a limited period but apparently make no attempt to penetrate cell walls or stomata.

Invasion of flowering stems

Since the mycelium of *E. typhina* travels rapidly up the pith of the flowering stem before the formation of stromata the possibility of its passage in the reverse direction was investigated. No infection had resulted from earlier attempts to insert spores and fragments of mycelium into the pith by means of a hypodermic needle and therefore an attempt was made to achieve it through the inoculation of cut stems such as would be found in a field after harvest. Flowering stems were cut at differing levels and conidia applied to the cut surfaces. Under conditions of high humidity these germinated well and the resulting hyphae entered the pith cavity and grew down the stem. Progress was slow at first ($\frac{1}{4}$ in. after 7 days) but became faster (1 in. after 14 days) and a hypha was traced for 3 in. after 17 days in one plant. This particular hypha had successfully passed through a node in its progress downwards (Pl. 19, fig. 4). Later in the season ascospores were substituted for conidia and similar behaviour was observed, though the rate of growth was rather slower, due possibly to the increased maturity of the host tissue. Hyphae grew in 6 of 28 stems inoculated with ascospores and in 15 of 38 stems inoculated with conidia.

Inoculations on a field scale were carried out in July 1953 by arranging jars of ripe perithecial stromata along drills of seeding cocksfoot, some of which was cut back as in normal harvesting, leaving about 6 in. of stubble. The cut surfaces were also lightly brushed with stromata. Adjoining rows were untreated and used as controls. In September the whole area was lightly cut back as a normal cultural measure. The results in 1954 were interesting in that for the first time stromata were obtained in artificially inoculated material. Infection in the rows ranged from 0 to 10% (av. 5.4%) and the distribution of the stromata was similar to that normally found in a light attack in a normal crop, i.e. single or small groups of 2-5 stromata, well scattered along the rows.

This result was confirmed in 1957 on potted plants after unsuccessful attempts to do so in plots. By using potted plants it was found that success-

ful penetration of the cut stems occurs only in a saturated atmosphere. It seems that such a condition must prevail for a few days for the successful establishment of the fungus in cocksfoot. If this is so, it suggests a reason for the failure of *E. typhina* to spread from infector plants to healthy neighbours when these are grown as spaced specimens. Under this system of cultivation the necessary degree of humidity is unlikely to be reached.

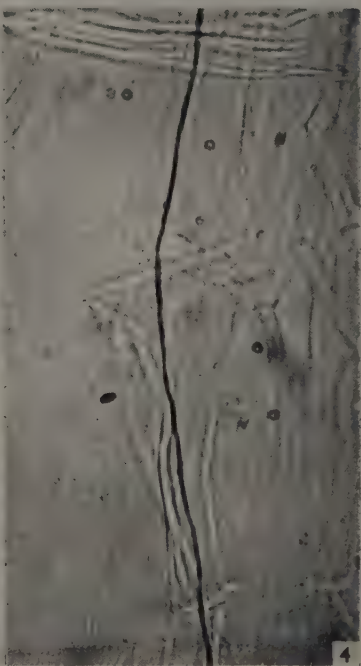
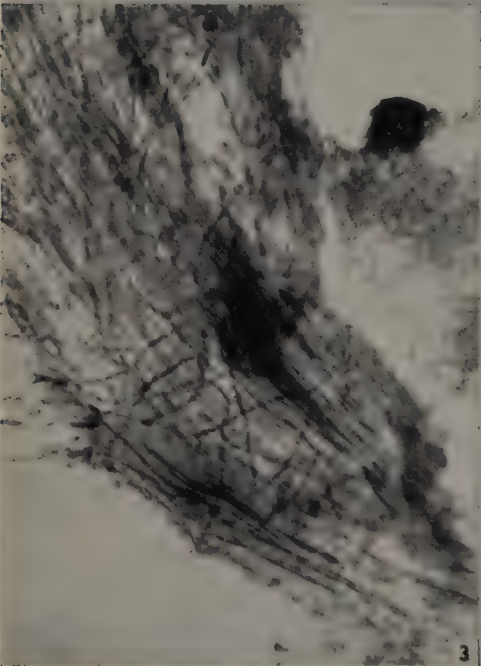
DISCUSSION

The evidence presented here suggests that the cocksfoot disease is not transmitted by the seed, but the discovery of the ability of hyphae from conidia and ascospores to enter the stubble through its cut ends and to establish themselves within the host with the production of stromata, provides a feasible explanation of the spread of the disease in the field. If field infection is in fact through the stubble the reasons can be seen for the rather unusual features of choke on this host. It would explain, for instance, the absence of stromata in the first harvest year but, at the same time, show how exceptional infections in such crops could arise. Such a case was seen by us in Northumberland, where a block of cocksfoot was sown in two parts, the first in March 1949 and the second in July. When the two areas were sampled in June 1950 the March sowing contained 7% infected heads but the younger July one only 0.1%. The difference may well have been due to the greater number of flowering stems produced in the older portion in its seeding year (1949) which would have been cut back by the stock grazing the field. The plants in the portion sown in July would be too young to produce flowering heads that year and so escaped infection.

For successful infection favourable conditions must prevail. Humidity appears to be the most critical factor, as under dry conditions the stubble dries out rapidly and the spores either shrivel and die or the host tissue becomes desiccated and unsuitable for penetration. The importance of the correct environment at harvest time may well determine the amount of disease in subsequent years.

Infection of the first-year crop is probably the result of inblown spores, but a few stromata in subsequent crops could cause the disease to spread rapidly under the right conditions. From observations on the longevity of ascospores it seems unlikely that infection from old stromata or plant debris can be important. Stromata appear to be relished by insects and other soil animals and many are destroyed in this way. The presence of these predators may have a bearing on the spread of the disease since we have found occasional stromata in control rows in the field and also in pot experiments where the controls have not been well isolated. We have failed, however, to induce infection in healthy plants by various wound techniques and the invasion of the pith of flowering stems remains the most probable source of field infection. The evidence presented in this paper supports such a conclusion.

The authors are indebted to the Agricultural Research Council for their financial support of this work. They also wish to thank the Director and



staff of the Welsh Plant Breeding Station, Aberystwyth, and Mr T. E. Miln of Messrs Gartons, Warrington, for providing names and addresses of cocksfoot growers.

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EXPLANATION OF PLATE 19

- Fig. 1. A panicle of cocksfoot partially suppressed by a conidial stroma of *E. typhina*.
- Fig. 2. Section of an aborted seed from such a panicle showing invasion by hyphae of *E. typhina*. $\times 120$.
- Fig. 3. Germinating conidia on stigma tissue. The hyphae do not penetrate the underlying cells. $\times 480$.
- Fig. 4. A hypha of *E. typhina* growing down the pith of a stem base of cocksfoot following inoculation of the cut surface of stubble. $\times 480$.

(Accepted for publication 13 October 1958)

NEW TECHNIQUES FOR ISOLATING SPORES OF HYMENOMYCETES

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(With 2 Text-figures)

Techniques for the isolation by micromanipulation of (i) basidiospore tetrads, (ii) oidia, random basidiospores and hyphal tips, are described. Both techniques dispense with hanging-drop cultures and reduce the need for sterile glassware to a minimum. They have been found suitable for isolating spores from most types of Hymenomycetes.

INTRODUCTION

The isolation of single spores from hanging-drop cultures supported by van Tieghem cells has been made relatively easy by the micromanipulators now available (de Fonbrune, 1949; Barer & Saunders-Singer, 1948). Although this method has proved ideal both for isolating endogenous spores, which require to be released by microdissection prior to isolation, and single random spores, it is not suitable for exogenous basidiospore tetrads which break up when hymenia are placed in a hanging-drop. Techniques evolved for isolating such spores have been described by Kniep (1922), Hannah (1924), Buller (1924) and Quintanilha (1933). More recently Papazian (1950), using these same techniques as a basis, described a method for isolating tetrads of *Schizophyllum commune*. We have found that this method has two disadvantages, namely (i) the need for special sterile apparatus, (ii) the inconvenience of incubating a section of gill prior to isolating the spores. Also Papazian's method of removing tetrads with a micro-needle makes the complete separation of all four spores from the basidium more difficult than their isolation with a micro-loop. The first technique described below overcomes these disadvantages. It was evolved for carrying out genetic work on *Collybia velutipes* but has been found suitable for isolating tetrads from species of most Hymenomycete families.

The second technique described below was developed in order to dispense with the unreliable method of making isolations from random spore platings. From such isolations it is not always possible to decide with certainty whether or not every colony arises from a single spore. In a heteromictic fungus, a dicaryotic colony would be assumed to have been formed by fusion of mycelia from two compatible spores. It has been found in *C. velutipes* that this assumption is invalid, since dicaryotic mycelia may occasionally arise from single spores (Bevan & Kemp, unpublished). The ease and certainty of the method described makes isolations from random platings no longer worth while.

THE ISOLATION OF BASIDIOSPORE TETRADS

The technique is illustrated diagrammatically in Fig. 1. A Petri dish is poured with suitable medium to a depth of about 0.4 cm. and, if possible, left for 24 hr. before it is used, so that the surface is dry. The agar is then cut into a block (*A*) with approximately 1.5 cm. sides and into a number of small blocks (*B*) with 0.75 cm. sides. A cross just penetrating the surface of the agar is cut on each small block with a flamed portion of a razor blade or fine needle.

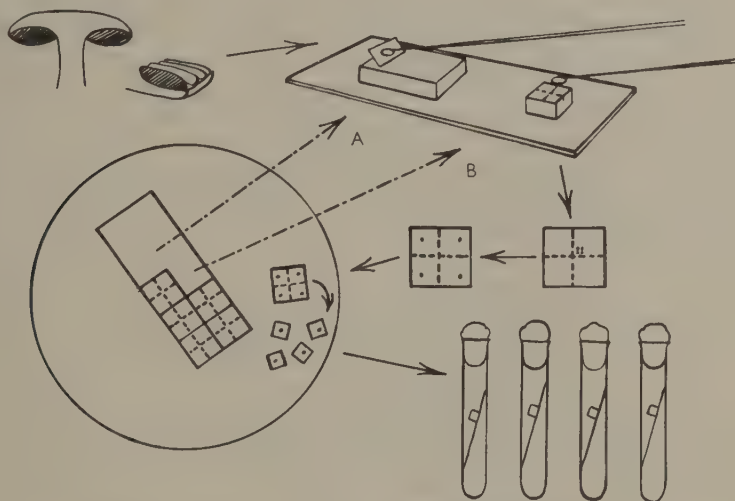


Fig. 1. Diagram illustrating the method of isolating basidiospore tetrads.

The larger block is then placed towards one end of a well-flamed glass slide and a portion or section of the hymenial surface bearing tetrads is placed on it at an angle of 45° , if necessary being supported by a small piece of agar. For species belonging to the Agaricaceae, a whole gill or part of one may be used, but for a typical member of the Polyporaceae a slanting section $50\text{--}100\mu$ thick is necessary. Sufficient light penetrates or can be directed on to a gill for tetrads to be isolated from the whole hymenial surface. When a section is used only those tetrads projecting beyond the surface can be easily removed. A tetrad is removed with a micro-loop, $10\text{--}15\mu$ in diameter, mounted in the micromanipulator. If the loop is dry when it touches a mature tetrad, the spores will stick on its outside, but if moist they will settle within it. All the spores are isolated from the basidium in one operation but can be removed separately if desired. The loop is then raised and the slide moved 2 or 3 cm. by the mechanical stage of the microscope so that one of the smaller blocks (*B*) can be placed under the loop in the centre of the field. The loop is lowered and the four spores deposited on this block in such a position, near the intersection of the two lines, that they can be found again immediately. Each spore is then picked up with the loop and placed in the centre of

one of the small squares and the whole block transferred back to the surface of the remaining agar in the Petri dish. A mounted portion of razor blade has been found ideal for this purpose. When the required number of tetrads has been isolated, the blocks are cut into four and each part put on a separate slope of nutrient agar.

THE ISOLATION OF OIDIA, RANDOM BASIDIOSPORES AND HYPHAL TIPS

This technique is illustrated diagrammatically in Fig. 2. A Petri dish of nutrient agar medium is poured to a depth of 0.5 cm. Towards one side of the dish an area 1×2 cm. is marked out on the surface of the medium with a fine needle. Within this the spores of hyphal tips to be isolated are smeared. The remainder of the medium is cut obliquely into four V-shaped strips, 0.5 cm. in surface width. These strips are then cut crosswise every 0.5 cm., so as to obtain a series of small blocks on each of which a spore or hyphal tip will be deposited. These V-shaped blocks are easier to remove from the plate than are blocks with vertical sides. In our experience, very little air-borne contamination results if the number of isolations per plate is limited to between 20 and 30.

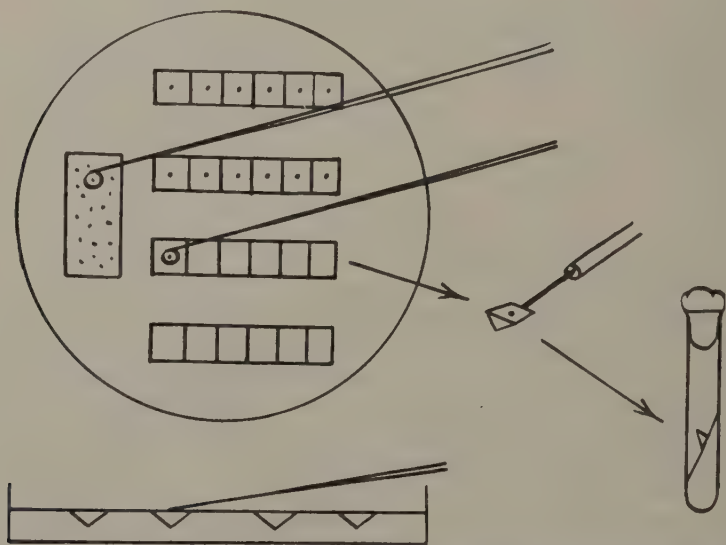


Fig. 2. Diagram illustrating the method of isolating random spores and hyphal tips.

The Petri dish is placed on the microscope stage and the micro-loop lowered within range of the smeared surface of the medium. The loop must be mounted in the manipulator so that at least half the plate can be reached without the shaft of the loop hitting the edge of the dish. When a spore or hyphal tip has been isolated within the loop the latter is raised and the dish moved manually so that one of the small blocks on which the isolate is to be deposited is in the centre of the field. For isolating hyphal tips a larger loop, approximately 20μ in diameter, is necessary.

In both techniques the following two precautions are worthy of note: (1) avoid freshly poured plates, as the spores are difficult to pick off the moist surface and the loop becomes blocked with agar; (2) use a clean loop: a blocked or dirty loop may be cleaned in chromic acid and washed in sterile distilled water.

We have found the most convenient magnification for isolating spores to be 225 diam., which is given by a binocular microscope having a $\times 1.5$ magnification with a $\times 10$ objective and $\times 15$ eyepieces. All the micro-loops which we use are made on a de Fonbrune microforge, using platinum filament wire 0.005 in. diam. and $\times 15$ eyepiece.

One of us (R.F.O.K.) is indebted to the Department of Scientific and Industrial Research for a research studentship.

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(Accepted for publication 14 October 1958)

DIRECT OBSERVATION OF *VERTICILLIUM* *ALBO-ATRUM* IN SOIL

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(With Plates 20-22)

The behaviour of *Verticillium albo-atrum* on inoculum fragments and on tomato roots in soil was studied directly by the use of glass-walled observation boxes.

While very limited and transient extension of free mycelium into soil occurred from certain food bases, the fungus was largely confined to those tissues which it initially invaded as a parasite. Sporulation occurred on inoculum fragments after their deposition in the soil, and also on infected roots following their death. The length and vigour of this sporulation phase appeared to be determined by the nutritional status of the base.

Since conidia may be produced abundantly over extended periods in soil, it is suggested that they play an important role in the epidemiology of diseases caused by *V. albo-atrum*. At least initially (during the sporulation phase), contact between host plant roots and diseased residues is not considered to be a necessary prerequisite of infection.

INTRODUCTION

Verticillium albo-atrum Reinke & Berthold is of wide geographical distribution and the cause of serious disease in a great number of economically important crops. Although it is amongst the most intensively studied of disease organisms, there is surprisingly little evidence concerning its mode of life in the soil. Garrett (1944) has summarized the available information, derived from indirect evidence, stating that 'a root does not become infectious to other roots in contact with it as soon as it becomes infected, because the fungus does not leave the vascular cylinder until the disease reaches its penultimate phase in the plant... No means of active spread through the soil except from plant to plant by root contact has been demonstrated for any fungus belonging to this group' (i.e. the vascular wilt fungi).

The investigations described below (which form part of a study of *Verticillium* wilt of the hop) provide direct observational evidence, in part supporting these general conclusions, and also indicating the importance of asexually produced spores in the life history of *V. albo-atrum*; this appears largely to have been overlooked in other studies of this pathogen.

EXPERIMENTAL METHOD

Observation boxes were prepared from four rectangular glass plates joined by adhesive polythene tape ($\frac{1}{2}$ in. wide) all round their top and bottom edges. The greatest rigidity of structure was obtained by placing

the plates so that one vertical edge was over and one under the edge of the adjoining plates. Boxes were prepared in a range of sizes but, for work with tomatoes, $2 \times 3 \times 1$ in. was found to be most convenient.

Each box was placed on a firm surface and approximately 2 cm. of soil added. This was very firmly compacted to form a solid soil 'floor' before boxes were filled with soil and lightly firmed. John Innes potting compost (sieved through $\frac{1}{16}$ in. mesh) was used throughout these experiments.

Tomato seedlings, of cultivar Kondine Red, were grown in seed-boxes up to the first or second mature leaf stage, and then lifted for transference to glass boxes. After cutting the two adhesive tapes joining two walls of the box, one plate, representing the observation surface, was swung open as a door. The seedling root system was arranged so as to be displayed over the exposed soil surface and the box was then resealed. This procedure assured the presence of adequate roots, including the tap root and main lateral roots, at the observation face (Pl. 20, fig. 1). After planting, boxes were wrapped in black tarred paper and stood on sand-filled trays.

Generally 14–21 days were allowed for the establishment of tomato plants in their boxes before further treatment. Plants were inoculated by the application of a conidial suspension or by embedding in the soil observation face fragments of hop bine infested with *V. albo-atrum* (bine inoculum). The latter method enabled inocula to be placed in any desired position relative to a root. The boxes were examined regularly, generally at weekly intervals, using a wide-field binocular dissecting microscope and incident light.

The pathogen

The isolates of *V. albo-atrum* used in these investigations were originally obtained from wilted hops and included both 'progressive' and 'fluctuating' strains (Keyworth, 1942; Isaac & Keyworth, 1948). In culture all isolates produced dark, resting mycelium, or 'Dauermycelien', which was always readily distinguishable from the microsclerotia formed by *V. dahliae* Kleb. Isaac (1949) has shown that these distinct morphological types are further distinguishable in both their physiological and pathological behaviour, and considers that they should be regarded as separate species.

EXPERIMENTAL RESULTS

Behaviour of Verticillium albo-atrum on inoculum fragments

Fragments of bine inocula, approximately 2×4 mm., were pressed into the soil observation face at various distances from visible roots. The subsequent behaviour of *V. albo-atrum* was similar on both naturally infected, and on sterilized and inoculated hop bine fragments. Both forms of inoculum were air-dried before use.

Generally 14–21 days elapsed before conidiophores of *V. albo-atrum* developed over the surfaces of the inoculum fragments; these always developed more abundantly from cortical tissues remaining attached to the bine fragment than from exposed wood surfaces. Conidiophores were usually quite typical in form, often bearing two or three whorls of phialides

and having blackened conidiophore bases. The density of sporulation varied from a locally rich 'turf' to scattered single conidiophores, but was always at its maximum shortly after the onset of sporulation. After the first flush of sporulation, which lasted for 7-14 days, conidiophores developed with decreasing frequency. Sporulation on bine inocula continued for 2-7 weeks and was not affected by the proximity or even by actual contact with host plant roots.

The production of conidiophores on the surface of inocula was generally the only visible sign of the pathogen. Occasionally, however, growth was rather more vigorous from certain inoculum fragments, and sporulation occurred on humus and mineral soil particles adhering or immediately adjacent to the inocula. At the same time hyphae, often bearing sporing structures, grew from the inocula across interstitial soil spaces to other soil crumbs or roots. When such 'aerial' hyphae came into contact with soil particles no further development was ever observed: when they contacted roots a few conidiophores frequently developed on the root surface at or near the points of hyphal contact. Conidiophores occurring on soil particles adjacent to inocula, on 'aerial' hyphae, and on distant roots were always reduced in size and simple in form, often bearing only three phialides or one. In the latter instance *Verticillium* hyphae were distinguishable from those of *Cephalosporium* only by the presence of more typical conidiophores nearer the food base. No trace of *Verticillium* was ever observed more than 2 mm. distant from the inoculum.

Although other fungi invariably developed on bine inocula, they were rarely evident before *Verticillium* sporulated and no obvious signs of antagonism were noted. The most constantly occurring (sporing) colonizer was *Stysanus* sp.: species of *Chaetomium*, *Penicillium*, *Gliocladium* and *Aspergillus*, and *Trichoderma viride*, were also common. Frequently the cortical tissues of inoculum fragments, together with the *Verticillium* growing on them, were devoured by sciarid larvae. *Verticillium* spore heads and the distal parts of the conidiophores were commonly eaten by Collembola and soil mites.

Following applications of conidial suspensions, no evidence of the presence of *Verticillium* in the soil was observed.

Behaviour of Verticillium albo-atrum on root surfaces after inoculation

The occasional and sparse development of simple conidiophores on the surfaces of roots where they were touched by 'aerial' hyphae, or were in contact with inoculum fragments, was an ephemeral phenomenon associated with the first 'flush' of sporulation and growth on inocula. These conidiophores never developed thick-walled and darkened bases, and were rarely recorded in two consecutive observations.

There were no signs of cortical colonization subsequent to root contact by 'aerial' hyphae, but at points where young, extending roots were in contact with inoculum fragments, dark mycelium occasionally developed on the root surface (Pl. 20, fig. 2). Dark mycelium rarely developed within 3 weeks of contact and did not spread extensively over the root surface, being more or less confined to the area of contact, where it remained

visible throughout the test period, i.e. for up to 18 weeks. Generally, however, dark mycelium was not seen on the roots of plants which later were found to be extensively infected.

The only external evidence of a marked reaction between tomato roots and *Verticillium* occurred when root tips grew into contact with inoculum fragments. Occasionally apical growth of the root was then arrested and all tissues within 3 or 4 mm. of the tip were killed, rapidly changing from glistening white to dull greyish brown. Following this reaction conidiophores developed sparsely from the root tip (Pl. 20, fig. 3). Such conidiophores developing after necrosis of root tips were typical, bearing two or three whorls of phialides and with darkened conidiophore bases. Tissues proximal to the dead tip remained apparently unaffected, and often the root branched behind the affected region and continued to grow normally.

Following applications of conidial suspensions, no external signs of root infection were observed.

Behaviour of Verticillium albo-atrum on roots after death of the host

Although infection of the tomato plants could be demonstrated by stem-base isolations made 6–8 weeks after both types of inoculation, there was no external evidence of widespread colonization of roots until after death of the host. Probably because of their low nutritional status (Roberts, 1943) tomato plants in observation boxes rarely showed symptoms of wilt, and, in the initial series of experiments, all plants were retained until normal death occurred at the end of the season.

Degeneration of the root system was first apparent when fine roots became dulled in colour and lost turgor; similar signs of tissue collapse then extended throughout the root system. Animal attack on roots often followed rapidly. Sciarid larvae, particularly, caused extensive destruction of the cortical tissues, and, in a few boxes, many fine roots were stripped to such an extent that only the vascular cylinders remained. Enchaetrid worms commonly invaded the inner cortical tissues of larger roots, causing them to collapse into flattened ribbons—the external cortex lying loosely round the stele.

Sporulation of *V. albo-atrum* on roots commenced shortly after the onset of root degeneration (Pl. 21, figs. 4, 5), conidiophores first occurring on fine roots. The fact that sporulation was not limited to roots in contact with inocula suggests that infections had been effected by water-dispersed conidia. Conidiophores also appeared early on the vascular tissues of roots decorticated by sciarid larvae. Sparse sporulation on fine roots was followed rapidly by sporulation on main lateral roots and, finally, on the tap root, on which conidiophores were often so profuse as to form a dense 'turf'. Sporulation was frequently accompanied by marked blackening of the cortical tissues, caused by the presence within them of dark resting mycelium. Dark mycelium was never noted on the *external* surfaces of roots after their death, but the bases of conidiophores usually became thickened and dark (Pl. 22, fig. 6).

Sporulation was always most extensive and vigorous shortly after its

commencement (as on inoculum fragments when first introduced into the soil) and later-formed conidiophores occurred with decreasing density. The density and rate of decrease of sporulation were closely related to the size of the root and to the rapidity of visible degeneration of its tissues. Sporulation on the tap root frequently continued long after it had ceased elsewhere on the root system.

In nearly all instances, *V. albo-atrum* was the first visible (spring) colonizer of the cortex. Secondary colonizers noted were species of *Stysanus*, *Penicillium*, *Gliocladium*, *Cephalosporium* and *Cladosporium*, and *Trichoderma viride*. Species of *Mucor* and *Mortierella* were observed occasionally, but, when present, their growth was profuse and their mycelium extended widely into the surrounding soil. The presence of these fungi did not noticeably affect the sporulation of the pathogen, the conidiophores of which grew through dense 'turfs' of other fungi, including *T. viride*. On agar plates, *V. albo-atrum* is markedly inhibited by a wide range of fungi and particularly by *T. viride*.

Conidiophores were confined to root surfaces, with rare exceptions when they occurred on soil particles adjacent to roots (Pl. 22, figs. 7, 8). Local colonization of soil was almost invariably restricted to soil particles surrounding large roots, and the extent of visible soil colonization was related to the size of the root from which it originated. Hyphae growing across soil spaces, and bearing simple 'trident' and cephalosporium-like conidiophores (Pl. 21, fig. 5), developed only from the largest roots. Visible mycelial extension into the soil seldom exceeded 2 mm., and sporulation on soil particles was always transient, being apparent only during the variably rich, first flush of sporulation following death of the host.

Throughout these observations considerable variation in the sporulation period was noted, and on rare occasions a second flush of sporulation occurred after the initial phase had ceased. The sporulation period generally extended over 3-7 weeks, with exceptional records of 10 weeks. Since prolonged sporulation always occurred on the largest roots, it is possible that this period would be lengthened considerably in plants growing under natural conditions and attaining more widespread and bulky root systems.

Effect of killing infected plants on the sporulation of Verticillium albo-atrum

Under glasshouse and laboratory conditions tomatoes were very tenacious of life and, even after heavy inoculation with *V. albo-atrum*, rarely died before the end of their normal season. Table 1 summarizes the results of an experiment in which root death was accelerated by the following treatments: (i) application of sodium chlorate (1 % solution) to the soil; (ii) removal of all above-ground tissues (decapitation); (iii) ring-barking 1 in. above soil level. The efficiency of these treatments in hastening host death and sporulation on the roots was in the order noted. Thus, after application of sodium chlorate, all aerial tissues collapsed within 5 days, roots were visibly degenerating within 14 days and sporulation of *V. albo-atrum* commenced in the ensuing week.

Table 1. *Sporulation of Verticillium albo-atrum on infected tomato roots following treatments differentially affecting their death rates*

Time after treatment (days)	Sodium chlorate	Decapitated	Ring-barked	None
10	—	—	—	—
20	+++	—	—	—
30	++	—	—	—
40	++	+++	—	—
54	—	++	—	—
60	—	++	+	—
70	—	++	+++	—
86	—	+	++	+++
94	Not recorded		+	++

Density of sporulation: +, low; ++, medium; +++, high.

The most striking results were produced by killing plants previously inoculated with conidial suspensions of *Verticillium*, for although no trace of the pathogen was discernible after inoculation and up to the time of host death, vigorous sporulation accompanied visible root degeneration. The development of conidiophores on nearly every visible root indicated that widespread infection had occurred.

DISCUSSION

Three main conclusions can be derived from the observations described. First, extensive sporulation of *V. albo-atrum* occurs in soil on infected plant parts following their death; secondly, mycelial extension into the soil surrounding such residues is probably very limited and ephemeral; and thirdly, there appears to be no widespread root-surface colonization phase prior to infection. These conclusions must necessarily be viewed with some caution as the method is subject to the limitation that hyaline mycelium growing in close contact with soil particles, or with root surfaces, would be indiscernible unless accompanied by sporulation or by thickening and darkening of the mycelium. Furthermore, the environment of roots at the observation face, being that of a permanently exposed soil profile maintained under moist conditions, is not strictly comparable to that in normal field soil. There is, however, considerable harmony between these observations and the results of other investigations (reviewed below) which suggests that they do truly reflect the behaviour of the fungus under more normal soil conditions.

Infection occurred following applications of conidia or the contact, or near contact, of roots with infected plant residues. The latter type of infection was signalled infrequently by the occurrence of dark mycelium on the root surface, at points of contact. Dark mycelium has also been noted on the surfaces of hop roots 10 days after root contact with cornmeal inoculum fragments (Talboys, 1958). The present observations provide no evidence that the formation of dark mycelium on the root surface is an essential stage in the process of root penetration. Indeed, it seems more probable that the thickening and darkening of limited, pre-existing, and indiscernible hyaline mycelium on root surfaces is occasionally a visible

expression of cortical penetration, and may be affected considerably by the type and nutrient status of the inoculum and by environmental conditions.

When saprophytic in soil, *V. albo-atrum* appeared largely to be confined to the debris of those tissues which initially it had invaded as a parasite. While free mycelial growth in the soil surrounding infected residues did occur, particularly immediately after their death, such soil colonization was an ephemeral phenomenon possessing an 'explosive' quality. Both vigour and extent of outward growth appeared to be determined by the type of base from which it originated; the observations suggested that the determining factor was associated with the nutrient status of the base. Even so, within the range of tissues studied, mycelial extension into the surrounding soil seldom exceeded 2 mm. From an experiment in which *V. albo-atrum* inocula contained in perforated glass tubes were buried in soil, prior to host-plant sampling for radial spread, Isaac (1953*a*) concluded that the pathogen was entirely incapable of spreading saprophytically through soil.

The apparent absence of a freely spreading mycelial phase in the life cycle of *V. albo-atrum* supports Garrett's (1947) conclusion that there is little prospect of controlling *Verticillium* wilt *within* susceptible crops by the direct influence of soil conditions. While considerable *indirect* effects may be exerted by soil treatment, these are more likely to induce a form of tolerance in susceptible hosts than freedom from infection. Thus, nitrogen-deficient hops showing no external symptoms were frequently found to be infected (Keyworth & Hewitt, 1948).

No indication of host stimulation of *Verticillium* was noted in the present studies: neither in the direction of hyphal growth (where this occurred), nor in position and extent of sporulation did *V. albo-atrum* appear to be affected by the proximity of host roots.

While transient soil colonization by mycelial extension occurred only from freshly killed and relatively succulent host tissues, and occasionally from dry inocula when introduced into soil, sporulation occurred on all types of tissues. The duration and density of sporulation, however, were related to the quantity and rapidity of degeneration of the tissues. It was an interesting feature of these observations that even the complexity of the conidiophore structure appeared to be related to the nutrient supply, for conidiophores tended to become progressively more simple as distance from the food base increased, and sporing hyphae traversing soil spaces were frequently cephalosporic in habit.

Roberts (1943) has demonstrated that the rate of spread of the disease from wilted to adjacent healthy tomato plants was greatly increased when the infected plants were killed by ring-barking. Partly on the evidence of these experiments, Garrett (1947) concluded that 'an infected plant does not become infectious to its neighbours until the penultimate phase of the disease, when the fungus emerges from the vascular cylinder, and develops on the outside of the infected root.' This conclusion also receives support from McKay's (1926) observation that *increased* incidence of wilt in potatoes resulted when infected plants were rogued. Although McKay reasoned that this was due to neighbouring plant roots being able to

grow into a heavily contaminated area of soil, Garrett (1956) points out that the fungus may emerge more rapidly from the vascular cylinders of broken portions of diseased root left in the soil after roguing, thus increasing the infectivity of the area of soil formerly occupied by the diseased plant. That the rapidity of spread of *V. albo-atrum* to neighbouring healthy plants may be related to the death rate of those initially infected has also been suggested by Isaac (1953*a*).

These observations by McKay, Roberts and Isaac may readily be explained in terms of the behaviour of *V. albo-atrum* observed in the present study, where visible deterioration of infected roots invariably foretold the onset of sporulation over their surfaces; this phenomenon would undoubtedly result (by the dispersal of conidia in soil water) in a rapid increase in the infectivity of a considerable volume of soil surrounding infected, dying plants. Perhaps the strongest circumstantial evidence supporting this conclusion can be derived from the fact that Isaac (1953*a, b*, 1957) was able to isolate *V. albo-atrum* from soil near the roots of infected tomato, antirrhinum and lucerne plants for a number of weeks following their deaths. That he was able to do so by means of the soil dilution plate method suggests that the fungus was very abundant in the soil in the form of conidia (Warcup, 1955) during this period.

Behaviour of *V. albo-atrum* similar to that on tomato roots has also been observed on roots of *Antirrhinum majus*, *Humulus lupulus* and *Senecio vulgaris*. Sporulation on infected plant residues in soil, over extended periods of time, is therefore likely to be of considerable importance in the life cycle of this pathogen. While a number of investigators have effectively used conidial suspensions as inocula (e.g. Noble, Robertson & Dowson, 1953; Roberts, 1943), and sporulation from the surfaces of diseased tissues is a known characteristic of the pathogen (McKay, 1926; Keyworth, 1942; Isaac, 1957; Sewell & Wilson, 1958), the role of conidia in the epidemiology of Verticillium wilt has largely been overlooked. The epidemiological significance of conidia would, of course, not necessarily be confined to their production from roots. In most hosts *V. albo-atrum* penetrates to nearly all aerial tissues and infected leaves are readily abscised. In such a crop as the hop, for example, large quantities of infected leaf material may be distributed by wind: also infected bine bases and shallow roots may be spread by cultivation implements (Keyworth, 1942). On coming to rest in the soil, water-dispersed conidia from all such residues may render large volumes of soil infective.

On the basis of these considerations it is possible to modify the suggestion made in turn by Roberts (1943), Garrett (1947) and Isaac (1953*a*), postulating contact between a healthy root and an infected residue as necessary for infection. Thus, although spread of wilt is related to the death of the infected host plant, and no means of *active* spread has been demonstrated, a volume of soil far greater than that occupied by its roots may become infective after its death. The dispersal of conidia in soil water can be expected to increase considerably the apparent infective capacity of a small inoculum fragment. As long as sporulation continues, a large volume of soil may be rendered infective; as sporulation declines, together presumably with the nutrient status of the diseased residue, the infective

soil volume also will decrease—finally to the size of the residue, when root contact may then become a necessary prerequisite of infection.

The writer thanks Drs R. V. Harris and J. E. Crosse for advice in the preparation of this paper, and Mr E. Yoxall Jones for the photographs.

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EXPLANATION OF PLATES 20-22

PLATE 20

- Fig. 1. Glass boxes used for direct observation of *V. albo-atrum* in soil and on plant roots. In the right-hand box, a glass plate has been swung open to expose the soil observation face.
- Fig. 2. Dark mycelium of *V. albo-atrum* on the surface of a tomato root growing alongside a fragment of infected hop bine.
- Fig. 3. The tomato root has grown into contact with an inoculum fragment (out of focus) and the tissue of the tip has been killed. A single conidiophore of *V. albo-atrum* is growing from the dead tissue.

PLATE 21

- Fig. 4. Dense sporulation of *V. albo-atrum* from an infected, fine tomato root following its death.
- Fig. 5. Sporulation of *V. albo-atrum* from an infected, dead, tomato tap root. A few conidiophores are growing from a small soil particle adhering to the root surface (centre). Simple sporing structures are present on hyphae growing from the root at bottom left and top right.



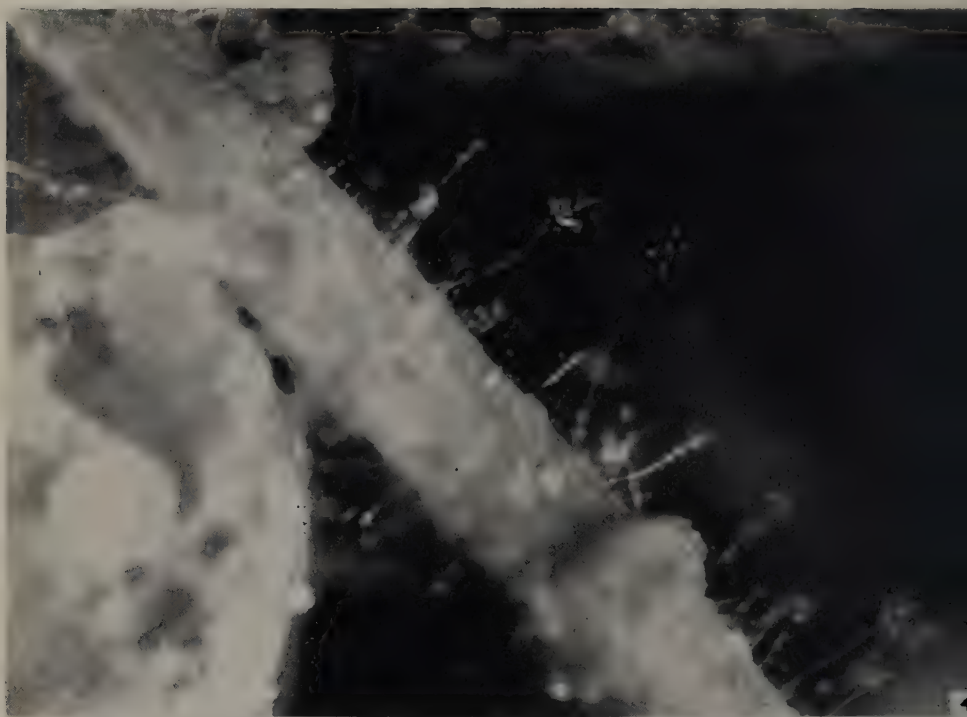
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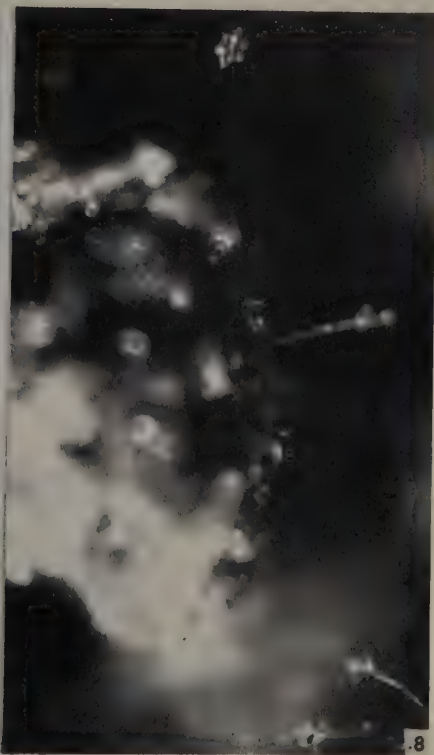




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PLATE 22

- Fig. 6. Sporulation of *V. albo-atrum* from a tomato root; the focus is adjusted to show the darkened conidiophore bases.
- Fig. 7. Conidiophores of *V. albo-atrum* on a sand grain wedged between two infected, dead tomato roots. Growth over soil particles from such fine roots was unusual.
- Fig. 8. Conidiophores of *V. albo-atrum* on a soil particle resting against the infected, dead tap root of a tomato plant.

(Accepted for publication 11 November 1958)

NECTRIA LUGDUNENSIS SP. NOV., THE PERFECT STATE OF *HELISCUS LUGDUNENSIS*

By JOHN WEBSTER

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(With 1 Text-figure)

Perithecia of a *Nectria* appeared in cultures of the aquatic hyphomycete *Heliscus lugdunensis*, and cultures derived from ascospores confirmed that the *Nectria* is the perfect state of the *Heliscus*. Mating experiments have shown that the fungus is heterothallic. These findings are discussed in relation to Ranzoni's earlier discovery that *Flagellospora penicillioides*, another aquatic hyphomycete, also has a *Nectria* perfect state.

Although over 40 species of aquatic hyphomycetes growing on submerged leaves have been described (Ingold, 1942, 1943*a-c*, 1944, 1949, 1952, 1956, 1958*a, b*; Ingold & Ellis, 1952; Ingold & Cox, 1957*a, b*; Ranzoni, 1951; Tubaki, 1957) only one, *Flagellospora penicillioides* Ingold, has been shown to have a perfect state. Ranzoni (1956) has described a *Nectria* which appeared in old cultures of *F. penicillioides*, and has produced evidence that the fungus is heterothallic. In the present paper similar findings are reported for a second aquatic hyphomycete *Heliscus lugdunensis* Sacc. & Therry (syn. *H. aquaticus* Ingold).

Leaves of holly (*Ilex aquifolium*) were collected from the bed of the River Porter below Forge Dam, Fulwood, Sheffield, on 18 June 1958, kept in shallow dishes of water for 2 days and then examined for aquatic hyphomycetes. Cultures were prepared from conidia of *Heliscus lugdunensis* by removing them with a sterile needle to plates of 2% malt-extract agar made up from stream water (pH 6.9) also collected from the River Porter. This medium had been found earlier to be superior for the growth of other aquatic hyphomycetes to 2% malt-extract agar prepared from tap water or distilled water; it will be referred to as 'stream water-malt agar'. The plates were stored overnight at 15° C. and spore germination took place readily. Transfers were made to slopes of stream water-malt agar (about 20 ml. of medium in 6 × 1 in. boiling tubes) on 21 June 1958, and stored in a glass cupboard on the ledge of a north-west-facing window in the laboratory. When one of the cultures was examined on 6 August, in addition to the whitish glistening drops of macroconidia, clusters of red perithecia were seen at the lower end of the tube. At this time

Legend to Fig. 1

Fig. 1. *Nectria lugdunensis*. A, L.S. perithecium; B, ascus and ascospores; C, microconidia; D, conidia from type specimen of *Heliscus lugdunensis*; E, conidiophores and conidia from a 10-day culture started from a single ascospore. (D and E drawn to same scale.)

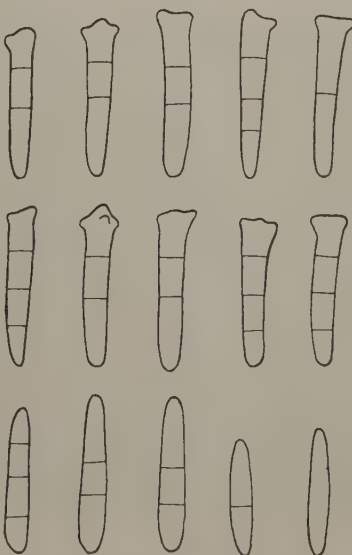
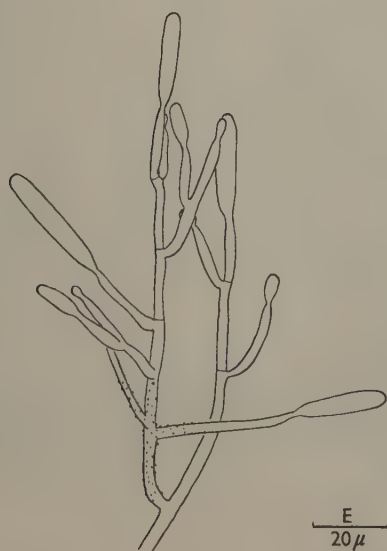
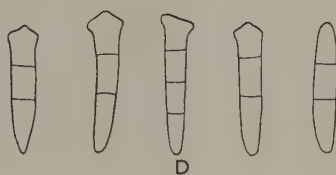
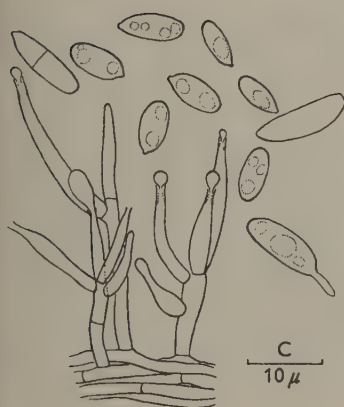
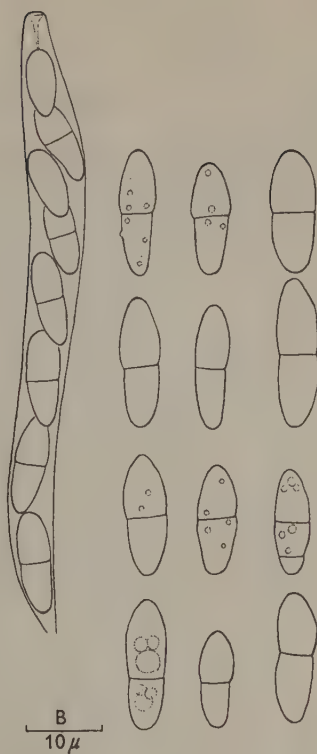


Fig. 1 A—E

perithecia when squashed were found to be immature, but when examined a week later asci and ascospores were discovered. Thirteen single ascospore cultures were prepared between 13 and 15 August, also multi-ascospore isolates, and others from single macronidia. All were grown on slopes of stream water-malt agar in boiling tubes and incubated as before. All produced a similar growth. Ten-day-old cultures were about 2 cm. diam. and were pale brown in the centre with a white margin, with similar coloration in reverse. A fluffy aerial mycelium with a tendency to aggregate into rope-like strands was formed. Clusters of macroconidia developed from phialides at the tips of aerial branches (see Fig. 1 E), and in older cultures the macroconidia coalesced to form cream glistening drops resembling the sporodochia of a *Fusarium*. The arrangement of the phialides with bundles of conidia at their tips is also reminiscent of *Fusarium*. The shape of the conidia varies from cylindrical-clavate to clove-shaped and the proportion of clove-shaped conidia increases with age. Mature clove-shaped conidia are up to 3-septate, hyaline, smooth and measure $32-42 \times 7-8 \mu$.

A feature of the older slope cultures is the production of microconidia at the upper end of the tube. They are formed in white sticky clusters at the tips of phialides which arise on branched conidiophores, often from rope-like aggregations of hyphae. The microconidia are elliptical to oblong or allantoid, smooth, hyaline, 0-1 septate, guttulate, $4-12 \times 2.5-4 \mu$, and capable of germination (Fig. 1 C). The macroconidia agree fairly closely with the description and figures of *Heliscus lugdunensis* (Saccardo, 1880, 1886), originally described from bark of *Pinus*. Fortunately material of this fungus is preserved in the Saccardo Herbarium at Padua, and through the kindness of Prof. C. Cappelletti I have been able to compare it with my material. The specimen (Cryptogames de Lyonnais-J.J. Therry No. 4419) consisted of pieces of *Pinus* bark bearing pustules with conidia resembling those described above, up to 3-septate, $32-38 \times 6-8 \mu$ (see Fig. 1 D).

Cultures derived from *Nectria* ascospores were sent to Prof. C. T. Ingold, who has examined them and stated that they agree with the species described by him (1942) from submerged alder leaves as *Heliscus aquaticus*. When this fungus was described he compared it with published figures and descriptions of *H. lugdunensis*. His material formed spores which were predominantly 2-celled, whilst those of *H. lugdunensis* were figured as 4-celled. This difference, and the difference in habitat, were given as reasons for describing the alder leaf fungus as a new species. Moreau & Moreau (1949) have described isolates of a fungus from submerged leaves which they ascribe to *H. lugdunensis*. Their descriptions match my material closely. They too have noticed the resemblance to *Fusarium* and have described microconidia. Moreau & Moreau have noted the great variation in the form and size of the macroconidia in their strain and write (p. 327): 'Ses variations sont parallèles à celles de l'*H. aquaticus* dont elle paraît distincte.' Having compared Saccardo's material with my own, which Ingold has agreed matches his *H. aquaticus* I have formed the opinion that the two are the same fungus. Ingold (personal communication) inclines to the same view.

Perithecia in culture. Single or in clusters of up to 50, bright red, with a slightly darker shining ostiole, broadly pear-shaped, ostiole rounded, perithecia darkening and becoming flattened longitudinally on drying, 200–300 μ broad and up to 350 μ high. Wall up to 50 μ thick, outer layers composed of thick-walled, pigmented, rounded or polygonal cells up to 20 μ across, inner layers composed of flattened or polygonal thin-walled cells.

Asci. Numerous, cylindrical, thin-walled, unitunicate, 8-spored 60–84 $\times$ 7–8 μ . The tip of the ascus is truncate and contains a refractile plug connected by a cytoplasmic strand to the uppermost ascospore.

Paraphyses. As long as the asci and up to 8 μ wide, thin-walled, septate, segments inflated.

Ascospores. Oblong or fusoid, slightly inequilateral, hyaline, smooth, uniseriate, 1-septate (occasionally 2-septate), faintly constricted at the septum, the upper segment slightly larger than the lower, 11–16 $\times$ 4–5 μ . Germination takes place by the growth of germ tubes from the end of one or both cells. Type material (a dried culture) has been deposited at the Commonwealth Mycological Institute, Kew, Surrey (I.M.I. 74951).

Nectria lugdunensis sp. nov.

Perithecia in vitro discreta vel caespitosa, rubra, cum ostiola fuscior et lucida et rotunda; perithecia pyriformia, in senectute lateraliter collapsa sunt, 200–300 μ lata, ad 350 μ alta. Muri ad 50 μ crassi, cellis exterioribus densis, coloratis, rotundis vel angularibus, ad 20 μ latis; interioribus vero angustioribus et muros tenues habentibus. *Asci* cylindrati et truncati, muros tenues habentes, unitunicati, octospori, 60–84 $\times$ 7–8 μ . *Paraphyses* muros tenues habentes septatae, segmentis inflatis, per totum ascum, ad 8 μ latae. *Ascosporae* leves, oblongae, fusoidae, inaequilaterales, hyalinae, uniseriatae, uniseptatae (vel rarius biseptatae), parvum constrictae, segmento superiore maiore quam inferiore, 11–16 $\times$ 4–5 μ . Status conidiatis *Heliscus lugdunensis* est. Ex foliis submersis putrescentibus *Ilicis aquifolii*, in rivulo Porter, prope Sheffield. Specimen typicale (cultura sicca) in Herb. I.M.I. (No. 74951) retinetur.

Mating experiments. A collection of thirteen single ascospore isolates was used in attempt to discover whether the fungus is heterothallic. Subcultures were made on slopes of stream water-malt-extract agar and to each was added a conidial suspension prepared from one isolate. The twelve paired cultures were incubated as before, and after 1 month flooded with sterile water. After 6 weeks perithecia were found in some of the paired isolates, and also in an isolate started from two ascospores, but not in any isolate started from a single ascospore or a single conidium. This evidence indicates that the fungus is heterothallic. Cultures of two interfertile strains have been sent to the Commonwealth Mycological Institute, and to the Centraalbureau voor Schimmelcultures, Baarn.

DISCUSSION

It is of interest that although over forty species of aquatic hyphomycetes have been described from decaying leaves of broad-leaved trees, many of which have been grown in culture, only two have been shown to have perfect states, both in the genus *Nectria*. Ranzoni's evidence that the perfect state of *Flagellospora penicillioides* is a *Nectria*, and the demonstration

that *Heliscus lugdunensis* also has a *Nectria* perfect state should stimulate search for further connexions. It is possibly significant that both conidial states are phialospore forms, and should it prove general that the perfect states are heterothallic a profitable line of investigation might be to make paired cultures of conidial isolates of other phialospore forms. It will also be of interest to search for perithecia in nature, for it is at present unknown whether these are formed on leaves or on twigs, and whether they discharge their spores under water. The role of the microconidia reported in both species is at present unknown, and though additional spore forms have been described for a number of aquatic hyphomycetes (for example the pycnosporos of *Clavariopsis aquatica* (Ingold, 1942) and the 'monilioid arthrospores' of *Heliscus longibrachiatus* (Ingold & Cox, 1957) these appear to be quite different from those found in *H. lugdunensis*. Microconidia similar to those of *H. lugdunensis* have been reported in *Fluminispora ovalis* Ingold (1958*b*). The possibility that microconidia might have a sexual function preceding perithecium formation should not be overlooked and the presence of such structures might provide a clue to further species with perfect states. The resemblance of the conidia of *Heliscus lugdunensis* to those of *Fusarium* has already been noted by Moreau & Moreau (1949) and by Ingold & Cox (1957*a*) and the demonstration of a perfect state within the genus *Nectria* provides further support for the suggestion, since *Fusarium* conidia are well known in various species of *Nectria*. Moreover, other aquatic species of *Fusarium* are known, e.g. *F. aquaeductum* (Radlk. & Rabenhorst) Sacc. (Radlkofer & Rabenhorst, 1863), which has been shown by Glück (1895, 1902) to have a perfect state, *Nectria moschata*.

It is a pleasure to thank Prof. C. T. Ingold and Mr C. Booth for helpful discussion, and Mr A. O. Hulton for the Latin diagnosis. I am also indebted to Prof. C. Cappelletti for the loan of type material, and to the Director, Royal Botanic Gardens, Kew, for permission to study literature. I gratefully acknowledge assistance from the University of Sheffield Research Fund.

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(Accepted for publication 3 November 1958)

Trans. Brit. mycol. Soc. **42** (3), 328-331 (1959).

PUCCINIA ANGELICAE-BISTORTAE AND *P. POLEMONII*, TWO RUSTS NEW TO BRITAIN

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(With 1 Text-figure)

A form of *Puccinia bistortae* with aecidia on *Angelica sylvestris*, and *Puccinia polemonii* are recorded for Britain.

PUCCINIA ANGELICAE-BISTORTAE KLEB.

Grove (1913) has written: 'The Puccinias on *Polygonum bistorta* are not yet well known.' He points out that among those with teleutospores having no apical papilla, two groups can be distinguished: (1) A form with an aecidium on *Conopodium* and its other spore stages on *P. bistorta*, which he records and describes for Britain as *Puccinia conopodii-bistortae*. The life history of this form had previously been demonstrated by Soppitt (1893, 1895). (2) Forms with aecidia on *Angelica sylvestris* and *Carum carvi* and teleutospores on *Polygonum bistorta* and *P. viviparum*. He suggests that the form with aecidia on *Angelica* should be called *Puccinia angelica-bistortae* Kleb. (= *P. cari-bistortae*), whilst the form on *Polygonum viviparum* should be called *Puccinia polygoni-vivipari* Karst.

Wilson & Bisby (1954) use the name *Puccinia bistortae* for the British material, and their annotations under this name show that they refer to *P. conopodii-bistortae* and *P. polygoni-vivipari*. They make no reference to the form with aecidia on *Angelica*, and according to Grove the aecidium on *Angelica* has not been found in Britain.

Discussion with Dr Wilson on the occurrence of a rust on *Polygonum bistorta* growing with *Angelica* near Sheffield led to the suggestion that infection experiments with these two hosts should be attempted.

On 21 May 1958 an area of waterlogged ground below Quiet Lane at Fulwood near Sheffield, where infected plants of *P. bistorta* had been seen in the previous year, was visited. Plants of *Angelica sylvestris* growing with the *Polygonum* bore proto-aecidia of a rust. Infected plants of *Angelica* were dug up and potted and uninfected plants of *P. bistorta* were also potted. Plants of *Angelica* and *Polygonum* were placed together under a bell jar on the following day, whilst plants of *P. bistorta* alone placed under bell jars served as controls. On 2 June 1958 uredospore and teleutospore pustules were found on those plants of *P. bistorta* enclosed with infected *Angelica*. No infections were observed on the controls. The artificially infected material of *P. bistorta* is preserved in the Mycological Herbarium of the University of Sheffield as Specimen 2258. The infection experiment was repeated on 7 June 1958 and uredospore pustules were seen on the infected

plants of *P. bistorta* on 17 June. These specimens are also preserved as Specimens 2255 (aecidia on *Angelica*) and 2256 (artificially infected *P. bistorta*). The experimental demonstration that the rust of *P. bistorta* near Sheffield has its aecidium on *Angelica* is supported by the field observations. The nearest *Conopodium* to the area of *P. bistorta* is about 200 yards distant, and no aecidia have been found after careful search.

A description of the specimens is given below: *Pycnidia*: on bright orange swellings on the petiole, midrib and veins of the leaflets, formed on the upper side of lesions on the leaves; pycnidia numerous, about $120\ \mu$ diam., with well-developed periphyses; pycnospores small, elliptical $2\text{--}3 \times 1\cdot5\text{--}2\ \mu$. *Aecidia*: associated with pycnidia on swollen lesions on the petiole and midrib, or on the lower side of leaf lesions, immersed, peridium poorly developed, margin not projecting, spores white, subglobose to elliptical or polygonal, $24\text{--}30 \times 20\text{--}24\ \mu$; thick-walled, verruculose. *Uredosori*: on the lower surface of the leaf blade of *Polygonum bistorta*, associated with yellow flecks visible on the upper side, appearing first as flat whitish blisters about 0.5 mm. diam., later as small powdery pustules, pale fawn in colour. Uredospores globose, delicately echinulate, $25\text{--}28\ \mu$, pale yellowish brown. *Teleutosori*: usually hypophyllous, very rarely on the upper leaf surface, scattered, at first mixed with uredosori, later discrete, small, flat, 0.5–1 mm. diam., dark brown. Teleutospores broadly oblong, scarcely constricted at the septum, shortly stalked, brown, smooth, rounded, germ pores non-papillate, $24\text{--}36 \times 18\text{--}23\ \mu$ (Fig. 1A).

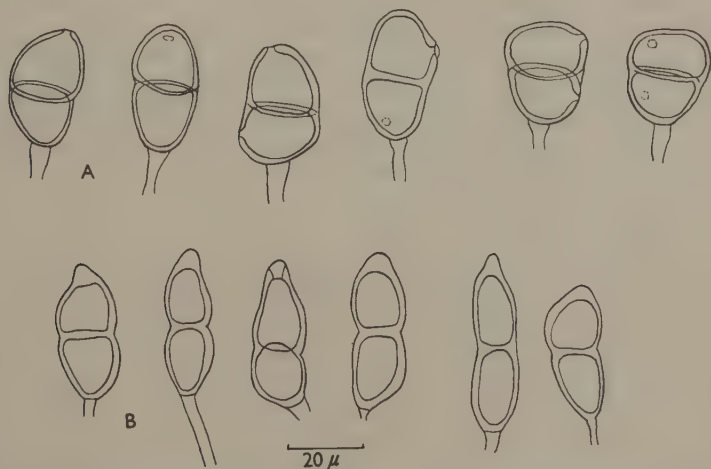


Fig. 1. A. *Puccinia angelicae-bistortae*. Teleutospores on experimentally-infected material of *Polygonum bistorta*. B. *Puccinia polemonii*. Teleutospores.

The measurements given above differ somewhat from those given by Grove (1913) for the form with aecidia on *Conopodium*. These are compared below:

	Aecidiospores	Uredospores	Teleutospores
<i>P. conopodii-bistortae</i> (Grove, 1913) (μ)	15–20	21–24	$28\text{--}42 \times 16\text{--}25$
<i>P. angelicae-bistortae</i> (μ)	$24\text{--}30 \times 20\text{--}24$	25–28	$24\text{--}36 \times 18\text{--}23$

Only the discrepancy between the sizes of the aecidiospores seems sufficient to deserve comment, but a morphological comparison between forms of the fungus on the different hosts seems desirable.

The nomenclature of rusts with aecidia on Umbelliferae and teleutospores on *Polygonum bistorta* varies from one author to another (e.g. de Candolle (1815), Klebahn (1896, 1898, 1899, 1900*a, b*, 1904), Fischer (1902, 1904), Juel (1899), Sydow & Sydow (1904), Jørstad (1932, 1940), Grove (1913), Wilson & Bisby (1954)). Some authors, such as Jørstad, have regarded all these rusts as forms of *Puccinia bistortae*, whilst others, such as Klebahn, have separated the various forms into species, *P. conopodii-bistortae*, *P. angelicae-bistortae*, etc. I do not wish to express an opinion on the nomenclature, but merely to record the presence in Britain of the form with aecidia on *Angelica*. The specimens are preserved in the Herbarium, Royal Botanic Gardens, Kew.

PUCCINIA POLEMONII DIETEL & HOLW.

Pigott (1958) has recorded, but has not described, collections of *Puccinia polemonii* on *Polemonium coeruleum* from three British localities: The Winnats, Monsal Dale and Taddington Dale in Derbyshire. The fungus was first described from America by Dietel (1893), and its distribution in other countries has been outlined by Jørstad (1932), and by Hylander, Jørstad & Nannfeldt (1953). The description below is based on a specimen from Monsal Head, 24 August 1957, collected by C. D. Pigott, and now in the Herbarium of the Royal Botanic Gardens, Kew.

Teleutosori mostly confined to the petioles, but spreading along the midribs of the leaflets, dark-brown, pulverulent. Teleutospores pale brown, smooth, oblong-cylindrical or clavate, not or slightly constricted at the septum, uppermost cell pointed and bearing a prominent cap of wall material; $34-48 \times 13-15 \mu$ (Fig. 1B).

In the original description Dietel describes two types of teleutospore 'the ones, principally in the centre of the sori, with a colorless or nearly colorless membrane thickened at the apex and with firm and long pedicels, germinating directly, are fusiform and little constricted at the septum before germinating; the others, with deciduous pedicels, germinating, as it seems, only after a period of rest, are yellowish brown in color, obovate or fusiform, distinctly constricted and surmounted with a conical hyaline papilla'. Jørstad (1932) writes: 'This character has not been clearly defined in the other descriptions seen, but is no doubt characteristic for the species, as it also holds true in Europe.' In the specimen described above the majority of spores had deciduous pedicels, but were prominently thickened at the apex of the spore.

I am grateful to Dr M. Wilson for helpful discussion.

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(Accepted for publication 6 November 1958)

OBSERVATIONS ON BRITISH SPECIES OF
PLEOSPORA. I

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(With 5 Text-figures)

Some new records of *Pleospora* species and their conidial states are made. *P. calvescens* forms conidia referable to *Microdiplodia henningsii*, and it is briefly compared with *P. betae*. *P. typhicola* forms a *Phoma* conidial state (possibly *P. typharum*), whilst *Pleospora scirpicola* produces no conidia in culture. *P. eximia* and *P. androsaces* are recorded for Britain; neither of them produced conidia in culture, but perithecia were formed freely.

I. *PLEOSPORA CALVESCENS* (Fr.) Tul. (Fig. 1)

Sphaeria calvescens Fries, Scleromycetae Sueciae, exs. no. 401 (1819-34).

Pleospora calvescens (Fr.) Tul., *Selecta Fungorum Carpologia*, 1863, 2, 266.

Cucurbitaria calvescens (Fr.) de Not., *Schema Spher. ital.* p. 215, 1861.

Pyrenophora calvescens (Fr.) Sacc., *Syll. Fung.* 2, 279, 1883.

Chaetoplea calvescens (Fr.) Clements, in Clements and Shear, *The genera of fungi*, New York, 1931.

Sphaeria echinella Cooke, *Handbook of British fungi*, 2, 906, 1871.

Microdiplodia henningsii Staritz, *Hedwigia*, 53, 161-163, 1913.

Perithecia of *P. calvescens* have been collected on various hosts but especially on dead stems of Chenopodiaceae near the sea during spring and early summer. The following description is based on a specimen on *Atriplex littoralis* collected on the sea bank at Clenchwarton Marsh, near King's Lynn, Norfolk, 6 January 1957 (Mycological Herbarium, University of Sheffield, no. 1910). Dimensions of the fungus on other hosts are listed in Table 1.

Perithecia: scattered or arranged in rows, seated on blackened areas of tissue, 170-200 μ diam., globose or depressed globose, dark brown to black in colour, at first subepidermal and penetrating the epidermis by a short neck, later as the epidermis is eroded the perithecia may appear superficial. The wall of the perithecium is 15-30 μ wide, composed of dark polygonal cells, and is surrounded at the base and sides by stiff dark-brown hyphae 4-6 μ wide. Dark hyphae penetrate the superficial layers of the host, causing a conspicuous darkening of the tissues. *Asci*: numerous, cylindrical, short-stalked, bitunicate, paraphysate, 8-spored, 80-130 $\times$ 10-11 μ . *Ascospores*: typically uniseriate or occasionally biseriate, fusoid-ellipsoid, inequilateral, with three transverse septa and with longitudinal septa in one or both of the central segments, or occasionally lacking

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longitudinal septa, constricted at the septa, especially at the middle, yellow to pale brown, $15-21 \times 7-8 \mu$.

Type material of *Sphaeria calvescens* Fr. (Scleromycetae Sueciae 401) was not available at Kew, the British Museum (Nat. Hist.), or the Muséum d'Histoire Naturelle, Paris, and has not been studied. We have, however,

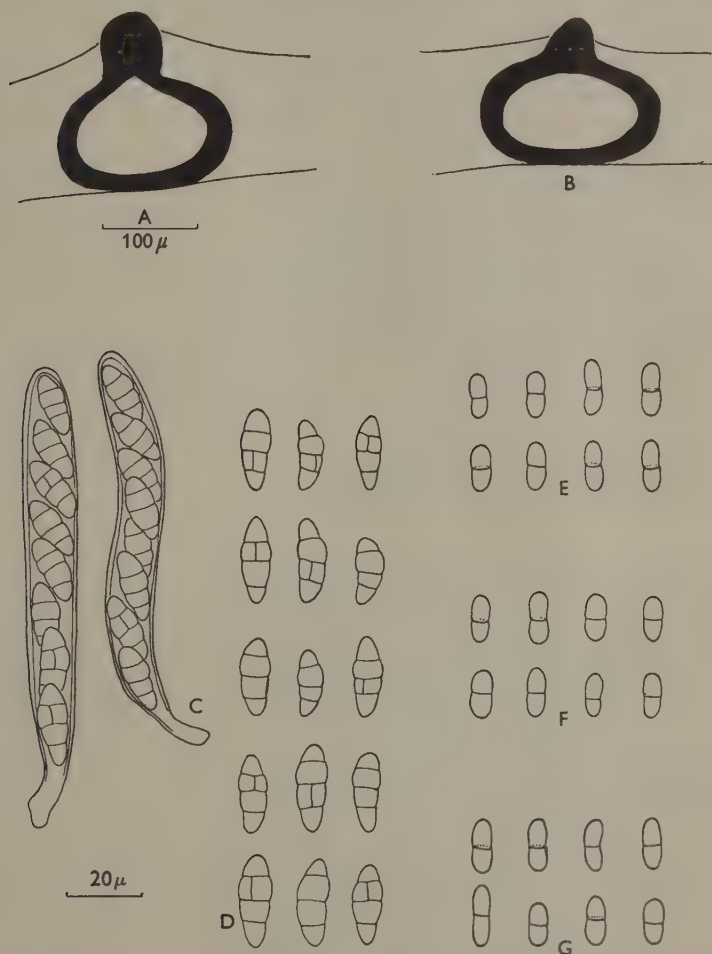


Fig. 1. *Pleospora calvescens*. A, L.S. perithecium on host (Herb. Sheffield 1910a); B, L.S. pycnidium on host (1910b); C, asci and ascospores (1910a); D, ascospores from type specimen of *P. bardanae* (Fungi Europaei no. 947 from Niessl's Herbarium); E, pycnosporos from host (1910b); F, pycnosporos from culture started from ascospores (1910a); G, pycnosporos from type specimen of *Microdiplodia henningsii* (Sydow Mycotheca Germanica no. 142). A and B to same scale; C-G to same scale.

examined a specimen, collected by Desmazières, from the Paris Museum, and this matches our own collections. According to Tulasne & Tulasne (1863) Desmazières had examined the Fries exsiccatum. Specimens collected by the brothers Tulasne (and thus authentic for the name *P. calvescens*) have also been matched with our material (see Table 1).

Pycnidia in culture: cultures were prepared from single ascospores of *P. calvescens* (Herb. Sheffield no. 1910a and from various other collections listed in Table 1). On oat agar slopes in diffuse light at room temperature the fungus grows rapidly, producing olive green aerial hyphae with paler, white or rose-coloured hyphae at the upper end of the slopes. The surface of the medium is black, and the medium coloured yellow. Pycnidia were observed within one month. They are black, globose, 240–360 μ diam. Pycnospores are produced by enlargement of the hyaline cells lining the pycnidium. Mature pycnospores are 1-septate, pale yellow, elliptical or oblong-cylindrical, sometimes slightly constricted at the septum, 9–13 $\times$ 4–6 μ (usually 4–5 μ).

Pycnidia on the host: similar pycnidia have been found on the host, sometimes in association with perithecia. In specimen 1910b they are scattered or arranged in rows, covered at first by the epidermis which is pierced by a papillate ostiole, depressed-globose, dark-brown to black, thick-walled, 130–240 μ diam., surrounded by dark brown hyphae. The pycnospores are oblong-cylindrical, rounded at the ends, straight or slightly curved, 1-septate, not or slightly constricted at the septum, pale yellow, 10–13 $\times$ 4–7 μ (mostly 4–5 μ). Cultures started from single pycnospores from the host formed cultures identical with those formed from ascospores.

Perithecia have not been found in culture.

Microdiplodia henningsii Staritz

The pycnidia described above matched the description of *M. henningsii* on *Chenopodium album*. The first reference to this fungus was made by Sydow & Sydow (1904), who published the name in a list of exsiccati, citing their Mycotheca Germanica no. 142. Descriptions of the fungus were given subsequently by Staritz (1913) and by Diedicke (1912). Our specimens have been matched with a portion of Sydow's Mycotheca Germanica no. 142 from Herb. Kew.

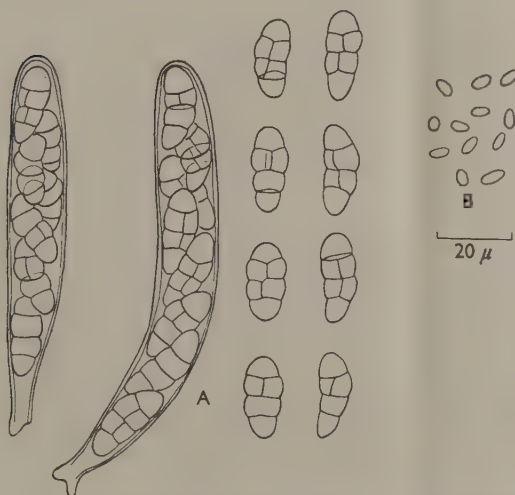
These findings agree with those of Tulasne & Tulasne (1863), who described 'pycnidia, which produce nothing but shortly ovate-oblong acrogenous stylospores, very obtuse at both ends, straight, bilocular, 10–13 μ long, 5–6 μ wide, smooth, fuscous, filled with oil, and supported on the very short crowded sterigmata that clothe the inside of the pycnidia'. We have seen such pycnidia on the Tulasne specimens. The Tulasnes also suggested that *Torula herbarum* and *Dendryphium comosum* might be the conidiophorous apparatus, but we have no evidence of this. Lind (1913) has repeated the suggestion.

Wehmeyer (1949) has remarked on the similarity of *P. calvescens*, common on Chenopodiaceae to *P. pellita* (= *P. papaveracea*) common on members of the Papaveraceae. Müller (1951) cites *P. pellita* as a synonym of *P. calvescens*, but Tulasne & Tulasne (1863) claimed that *P. pellita* was the perfect state of *D. penicillatum* (Corda) Fr., and this claim has been supported by the cultural work of Girzitska (1928) and various subsequent authors. We wish to reserve judgement about *P. bardanae* Niessl which also resembles *P. calvescens*. We have matched our material with type

Table 1. *Dimensions (μ) of asci, ascospores and conidia of Pleospora calvescens on various hosts and in cultures on oat agar*

Herb. no.	Host	Asci	Ascospores	Conidia	Culture conidia
Herb. Sheffield 1910a	<i>Atriplex littoralis</i>	80-130 $\times$ 10-11	15-21 $\times$ 7-8	—	9-13 $\times$ 4-5
1954a	<i>Reseda luteola</i>	90-106 $\times$ 12-14	18-27 $\times$ 7-10	—	Failed to fruit
1955	<i>Chenopodium album</i>	90-106 $\times$ 10-12	17-20 $\times$ 7-8	—	13-14 $\times$ 4-5
1959a	<i>A. littoralis</i>	90-116 $\times$ 10-12	17-22 $\times$ 7-9	—	10-14 $\times$ 4-5
2002a*	<i>A. littoralis</i>	110-120 $\times$ 10-11	18-21 $\times$ 7-9	—	—
2003a*	<i>C. sp.</i>	100-104 $\times$ 10-12	17-21 $\times$ 7-8	—	—
2004a*	<i>C. album</i>	90-110 $\times$ 10-12	17-21 $\times$ 7-9	—	—
Herb. Mus. Paris (coll. L. & R. Tulasne 23 April 1858)	<i>C. sp.</i>	80-110 $\times$ 10-12	15-20 $\times$ 6-8	—	—
Pycnidia:					
Sydow Mycotheca Germanica 142	<i>C. album</i>	—	—	10-14 $\times$ 4-6	—
Herb. Sheffield 1910b	<i>A. littoralis</i>	—	—	10-13 $\times$ 4-5	10-16 $\times$ 4-5
1954b	<i>R. luteola</i>	—	—	9-10 $\times$ 3-4	9-12 $\times$ 3-4
1959b	<i>A. littoralis</i>	—	—	10-14 $\times$ 4-5	10-14 $\times$ 4-5
1959b*	<i>A. littoralis</i>	—	—	9-13 $\times$ 4-5	—
2003b*	<i>C. sp.</i>	—	—	10-15 $\times$ 4-5	—
2004b*	<i>C. album</i>	—	—	9-13 $\times$ 4-5	—
2005*	<i>Beta vulgaris</i>	—	—	10-14 $\times$ 4-5	—
2006*	<i>C. sp.</i>	—	—	10-12 $\times$ 4-5	—

* Not grown in culture.

Fig. 2. *Pleospora betae*. A, asci and ascospores (2076); B, pycnosporos from 5-day-old culture on maize-extract agar.

specimens of this fungus from Herb. Kew, British Museum (Nat. Hist.) and Niessl's own Herbarium at Munich (specimens of Rabenhorst's *Fungi Europaei* no. 947). In spite of the closely similar morphology we would prefer to await culture studies based on material on *Arctium*, the original host, before assigning this species to synonymy.

2. *PLEOSPORA BETAE* BJÖRLING (Fig. 2)

Through the kindness of Dr C. E. Cornford (Cornford, 1958) we were sent material of *P. betae* on dead stems of wild beet *Beta vulgaris* (Herb. Sheffield no. 2076). We were immediately impressed by the similarity of this fungus to *P. calvescens*, but cultures from single ascospores produced the *Phoma betae* conidial stage within 5 days on maize-extract agar (Björling, 1944), as shown in Fig. 2. The ascospores of *P. calvescens* tend to be more fusoid and narrow, whilst those of *P. betae* are slightly wider and the end cells are more rounded (20 ascospores of *P. betae* measured $20-26 \times 8-10 \mu$). The asci of *P. betae* are also slightly wider than those of *P. calvescens*, $100-120 \times 13-14 \mu$ in the specimen examined. However, there is considerable overlap in dimensions, and recourse to cultures might be necessary to separate collections of the two on *Beta*. It is of interest that we have collected *P. calvescens* perithecia and pycnidia on *Beta vulgaris* (Herb. Sheffield no. 2075).

3. *PLEOSPORA ANDROSACES* FUECKEL, Symbolae Mycologicae, Nachtrag 3, 19, 1876 (Fig. 3)

Pyrenophora androsaces (Fuckel) Sacc., *Sylloge Fungorum*, 2, 284, 1883.
Pleospora fuckeliana Niessl, *Verh. naturf. Ver. Brünn*, 14, 34, 1876.

Perithecia of this fungus have been collected on dead leaves of *Silene acaulis*, both at Cwm Idwal near Capel-Curig, on 15 April 1957 (Herb. Sheffield no. 1962), and at Cwm Glas, North Wales (Herb. Sheffield no. 2080). The description is based on specimen 1962.

Perithecia: immersed, dark-brown to black, small globose, $160-180 \mu$ diam.; walls thick, composed of dark-brown polygonal cells $9-18 \mu$ wide. The upper part of the perithecium and ostiole are surrounded by divergent, tapering, septate, dark-brown setae up to 200μ long and about 8μ wide at the base. *Asci*: few (about 5 per perithecium), broadly clavate to elliptical, bitunicate, 8-spored, $130-146 \times 36-41 \mu$. *Ascospores*: biseriate, elliptic-fusoid, divided by a constriction across the middle into a wider upper half and a narrower lower half with 7 transverse septa, dark reddish brown when mature, $46-54 \times 20-24 \mu$. The spore is surrounded by mucilage and the epispore is sculptured into an irregular network of hyaline striations separating dark brown pigmented areas. The pattern of the striations apparently bears no relation to the segmentation of the spore.

Fuckel (1876) has cited Fungi Rhenani no. 2650, on *Androsace helvetica*, as the type specimen. A portion of this exsiccatum has been examined from Herb. Kew, and matches our specimens. Niessl (1876) cited the same specimen as the type of his *Pleospora fuckeliana*, which must therefore rank as a synonym of *P. androsaces*. There are various records of the fungus on *Silene acaulis* (Niessl, 1876; Winter, 1887; Müller, 1951). Winter (1887) has claimed that Fuckel's specimens, of which he had seen several, were growing on *S. acaulis*. The specimen in Herb. Kew has been compared with European species of *Androsace*, but does not match any, and we

concur with Winter's statement that the host is *S. acaulis*. Although the name *P. androsaces* is inappropriate it is no justification for accepting Niessl's name *P. fuckeliana*.

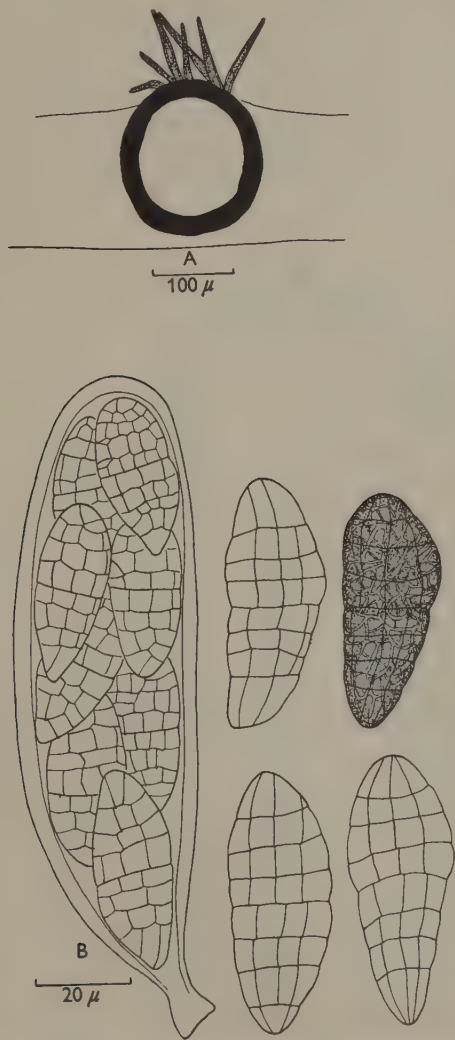


Fig. 3. *Pleospora androsaces*. A, L.S. perithecium on host (1962); B, ascus and ascospores from host

Cultures: ascospores germinated readily overnight and cultures were prepared from single ascospores. On oat agar slopes the fungus grows rapidly, forming a white aerial mycelium at first, which later develops patches of dark-brown hyphae. Perithecia were formed on oat and maize agars within 2 months in all cultures, including those derived from single ascospores. The perithecia, asci and ascospores resembled those on the

host plant. No conidia have been found. Cultures have been sent to the Commonwealth Mycological Institute and the Centraalbureau voor Schimmelcultures, Baarn, Holland.

4. *PLEOSPORA EXIMIA* REHM. *Mitt. naturf. Gesellsch. Thurgau*, **12**, 82, 1896 (Fig. 4)

Two collections of *P. eximia* have been made: Herb. Sheffield 1961 on spines of *Ulex europaeus*, Rhos Colyn, Anglesey, 16 April 1957, and Herb. Sheffield 2007 on dead stems of *Cynoglossum officinale*, Castle Martin, S. Wales, 27 June 1957. The following description is based on specimen no. 2007.

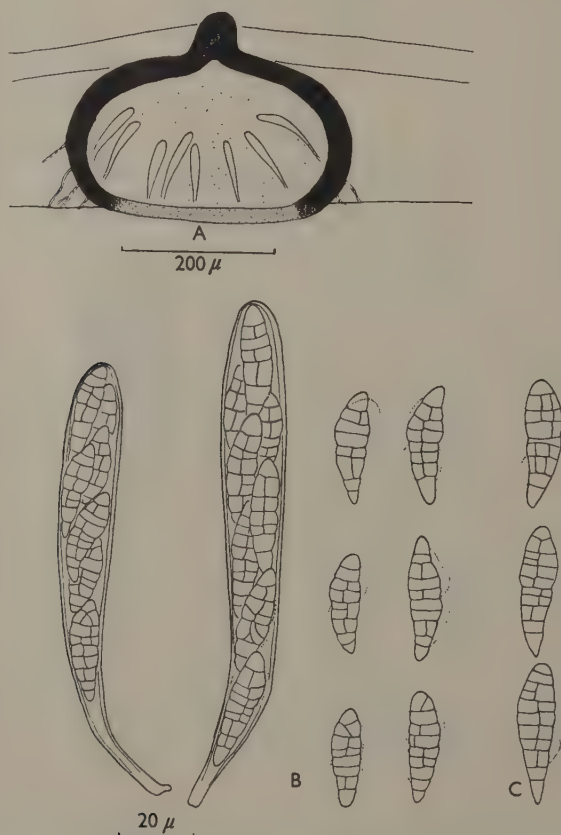


Fig. 4. *Pleospora eximia*. A, L.S. perithecium on host; B, asci and ascospores (2007); C, ascospores (1961).

Perithecia: subepidermal, depressed globose, up to $350\ \mu$ diam. and $200\ \mu$ high, pale-brown with a black cylindrical projecting neck composed of a bundle of parallel dark septate setae up to $80\ \mu$ long and about $4\ \mu$ wide. The wall of the perithecium is about $25\ \mu$ thick and composed of brown

polygonal cells above and hyaline cells at the base. The sides of the perithecia are clothed with yellow septate hyphae $2-3\mu$ wide. *Asci*: numerous, club-shaped, bitunicate, containing up to 8 spores, $14-120 \times 13-15\mu$, separated by numerous filiform paraphyses. *Ascospores*: biseriate in the upper part of the ascus, uniseriate below, boat-shaped, straight or curved inequilaterally, typically with 7-8 transverse septa, occasionally with 9. The spore is divided unequally by a constriction at the median transverse septum into a broader rounded upper half and a sharply tapering lower half. The central segments are further divided by one (or rarely two) longitudinal septa; the end cells are undivided. A characteristic feature of the spore is a mucilaginous envelope which is constricted in the middle of the spore. Mature spores are yellow to pale-brown and measure $28-35 \times 8-10\mu$ (excluding the mucilaginous envelope). Spores from specimen no. 1961 measured $23-36 \times 8-10\mu$.

Cultures: (2007): on oat agar slopes cultures derived from ascospores grow slowly, producing white aerial hyphae. Perithecia were found after 3 months in cultures started from multi-ascospore inocula. No conidia have been seen.

5. *PLEOSPORA TYPHICOLA* (COOKE) SACC. (Fig. 5)

Sphaeria (*Pleospora*) *typhaecola* Cooke, *Grevillea*, **5**, 121, 1877.

Pleospora typhicola (Cooke) Sacc, *Reliqu. Libert.* **2**, no. 152.

Clathrospora typhicola (Cooke) von Höhnelt, *Ann. mycol. Berl.*, **16**, 89, 1918.

Pyrenophora typhicola (Cooke) Müller, *Sydowia*, **5**, 256, 1951.

Perithecia of *P. typhicola* have been collected on dead leaf sheaths and stems of *Typha latifolia* and *T. angustifolia* from April to June. Cultures started from ascospores yield pycnidia possibly referable to *Phoma typharum* Sacc. The description below is based on Herb. Sheffield no. 2083, on *T. latifolia*, Sharnbrook, near Bedford, 13 April 1958.

Perithecia: large, black, globose or irregularly globose, single or in pairs, developing beneath the epidermis, and later becoming almost superficial, $280-480\mu$ diam., surrounded at the base by sparse dark-walled hyphae. Wall $40-80\mu$ thick; outer layers composed of dark-brown thick-walled flattened polygonal or rounded cells up to 15μ across; inner layers of hyaline thin-walled cells. The perithecia are visible between the veins as black dots, representing the upper wall of the perithecium, with a small papillate smooth ostiole penetrating the epidermis, and lying flush with the surface or projecting only very slightly. On stems, especially of *T. angustifolia*, the perithecia may project prominently. *Asci*: few in number, cylindrical or broadly clavate, rounded above, shortly stalked, bitunicate, 8-spored, $200-240 \times 44-48\mu$, separated by narrow septate paraphyses. *Ascospores*: irregularly biseriate, oblong, rounded at the ends, with three transverse septa and a longitudinal septum running down the length of the spore in one plane. When viewed from the side the longitudinal septum may not be visible. Each cell of the spore is rounded and may separate slightly from adjacent cells. Mature spores are hyaline to

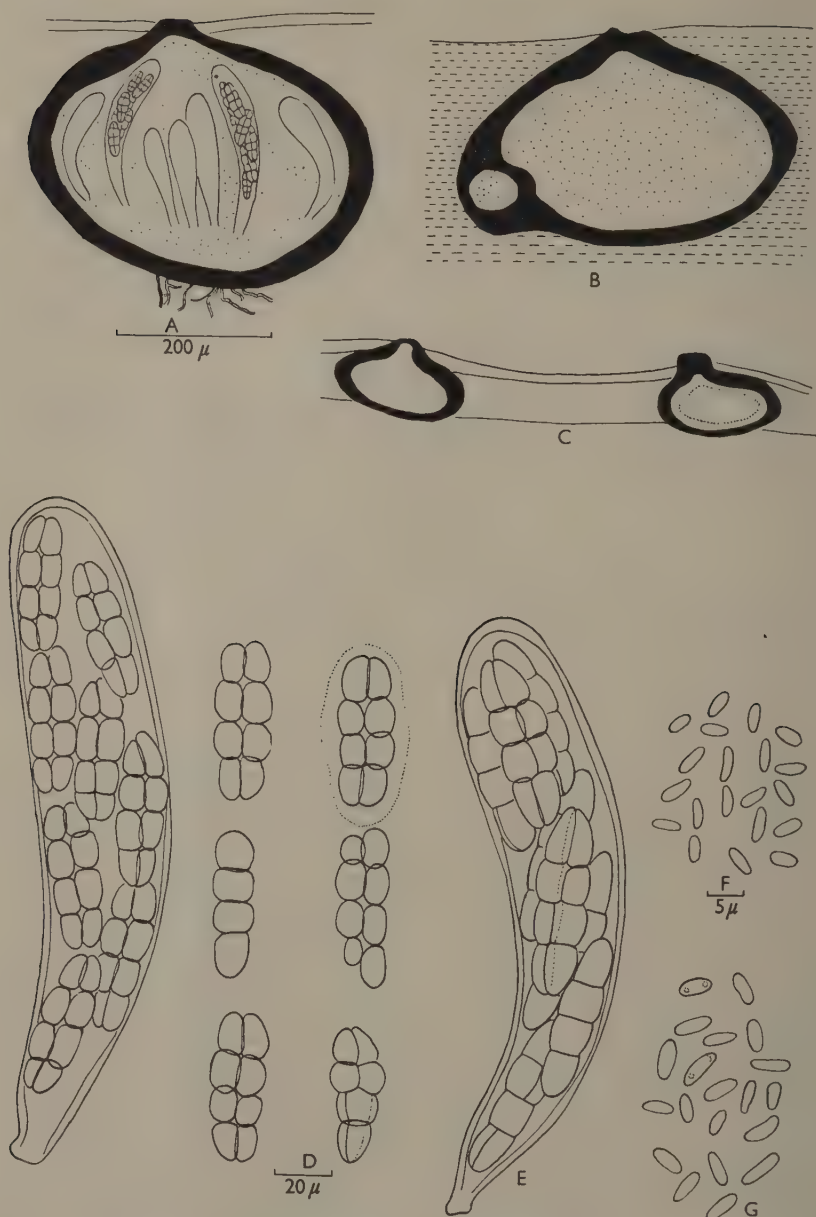


Fig. 5. *Pleospora typhicola*. A, L.S. leaf sheath of *Typha angustifolia* and a perithecium (2265a); B, section of a culture derived from a single ascospore (2083) showing a *Phoma* pycnidium; C, L.S. stem of *T. angustifolia* and a pycnidium (2265b); D, ascus and ascospores (2083); E, ascus from Plowright's *Sphaeriae Britannici* labelled '*Macrospora scirpi*'; F, Pycnospores from a culture derived from a single ascospore (2265a); G, Pycnospores from *T. angustifolia* (2265b). A–C to same scale; D, E to same scale; F, G to same scale.

yellowish brown, $46-56 \times 18-24 \mu$, and surrounded by a prominent continuous gelatinous sheath (Fig. 5D).

Cooke (1877) cited as a specimen of his *Sphaeria typhaecola* a specimen from Plowright's Sphaeriae Britannici labelled *Macrospora scirpi* on *T. angustifolia*. There is a Plowright specimen labelled *Macrospora scirpi* on *T. latifolia*, North Runcton, May 1873, bearing Cooke's Herbarium stamp, in Herb. Kew. In spite of the different host species we regard this specimen as authentic for *S. typhaecola*, which matches our collections (see Fig. 5E).

Cultures have been prepared from specimen 2083 and from two other collections of *T. latifolia* and *T. angustifolia*. Ascospores germinated overnight producing germ tubes from any cell. Single ascospore isolates were transferred to oat-agar slopes, and on this medium the fungus grew rapidly, producing a dark olive-green mycelium with large patches of white surface hyphae. The substratum was also coloured olive-green, and in some isolates purple shades were detected. Pycnidia were found within 6 weeks at the surface of the medium.

Pycnidia in culture: single or in groups, globose, or depressed globose, dark-brown, $120-240 \mu$ diam. The wall of the pycnidium is $15-30 \mu$ thick, composed of dark brown, polygonal cells up to 10μ across, and is surrounded by stiff dark hyphae $3-5 \mu$ wide. The pycnospores arise from a layer of isodiametric hyaline thin-walled cells $4-5 \mu$ diam., but the details of spore formation are difficult to interpret even at high magnification. Mature pycnospores are hyaline, smooth, cylindrical to elliptical, $4-6 \times 2-2.5 \mu$.

Pycnidia on the host: similar pycnidia have been collected on both *T. latifolia* and *T. angustifolia* growing either singly or with perithecia of *P. typhicola*. Cultures started from pycnospores from these pycnidia matched cultures started from ascospores. The description is based on specimen 2265b, on *T. angustifolia*, which has been grown in culture.

Pycnidia subepidermal, raising the epidermis and piercing it with a small blunt ostiole, pale brown, ellipsoidal, wall about 10μ thick, composed of flattened dark polygonal cells up to 10μ across, and lined by smaller hyaline cells from which the pycnospores arise; variable in size, but commonly $150-200 \mu$ diam. The pycnospores are pink in mass, cylindrical to elliptical or slightly curved, hyaline, $5-6 \times 2-2.5 \mu$.

The pycnidia are possibly identical with *Phoma typharum* Sacc., but Saccardo's description (*Syll. Fung.* 3, 163) gives no measurements, and in the absence of type or authentic material it is impossible to be certain of the identity of Saccardo's material. However, our specimens match descriptions under this name by Grove (1935). Saccardo (1884), Trail (1887), Allescher (1901) and Grove (1935) have all cited Fuckel's statement (1869) that the perfect state of *P. typharum* is *Leptosphaeria typharum*, but Webster (1955) has shown that *L. typharum* is the perfect state of *Hendersonia typhae*.

6. *PLEOSPORA SCIRPICOLA* (DC.) KARST.

We have made several collections of perithecia of this fungus on *Schoenoplectus lacustris* from the Lake District, and our material matches closely

the descriptions and figures by Ingold (1955). We have grown the fungus in culture but no conidial state has been found. Large black sclerotia, which may represent perithecial primordia were found in 2-month-old cultures on oat agar. Lind (1913) claims, without giving evidence, that the conidial state is *Clasterosporium scirpicola*.

It is a pleasure to thank the Director, Royal Botanic Gardens, Kew, for permission to study specimens and literature, and Prof. R. Heim and Dr E. Poelt for the loan of specimens. We are also indebted to Mr R. A. Blakelock of Kew for help with the European species of *Androsace*. The work was assisted by grants from the University of Sheffield Research Fund and the Gulbenkian Foundation, for which we are most grateful.

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(Accepted for publication 15 November 1958)

Trans. Brit. mycol. Soc. **42** (3), 343-353 (1959).

STUDIES OF FUNGI IN A *CALLUNA*-HEATHLAND SOIL

I. VERTICAL DISTRIBUTION IN SOIL AND ON ROOT SURFACES

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(With 3 Text-figures)

The distribution of fungi on root surfaces and in the soil horizons of a *Calluna*-heathland podzol were investigated by plating methods. No seasonal variation in the occurrence of fungi was apparent. The total number of species decreased with depth, but a few species were relatively abundant in the subsurface horizons. The occurrence of fungi on root surfaces was related to the soil horizon rather than to the root type. A few fungi were of constant occurrence and were considered to be characteristic species. A very stable fungus population was found to occur in the immediate subsurface horizon. The deeper, illuviated horizons and parent subsoil contained few fungi, of which the commonest were dark-coloured, non-sporing mycelia.

INTRODUCTION

Although attention was first drawn almost 30 years ago (Ling-Young, 1930) to the importance of studies of the fungal flora within natural plant communities, Warcup's (1951) investigations, employing the soil plate method, were the first convincing demonstration of the value of synecological studies of such habitats. Most earlier work has dealt with agricultural soils and, while yielding information of particular importance, has added little to our understanding of the general ecology of saprophytic soil fungi. The reason for this prolonged and relatively sterile period (from the ecological viewpoint) may lie in the fact that the dilution plate has been the standard method of isolation. Many criticisms of this method have been made, of which the most important are that the majority of fungal colonies originate from spores and that those spores which do germinate are determined by the isolation medium and degree of dilution employed (Brierley, Jewson & Brierley, 1927; Chesters, 1948, 1949; Chesters & Thornton, 1956; Cohen, 1950; Garrett, 1951; Harley & Waid, 1955; Warcup, 1955).

Clearly, any attempt to characterize a soil fungal community must be based on a determination of the active mycelial constituent of that soil (Chesters & Thornton, 1956; Harley & Waid, 1955). The fact that Warcup (1951) was able to demonstrate fairly marked differences between fungal populations in a range of different grassland soils, suggests that the species

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selected from these soil plate studies as being 'constant' were probably active members of the fungal communities. On the available evidence it seems that the soil plate may provide a useful and simple routine isolation method for synecological studies of soil fungi.

The present work is concerned with the distribution of fungi in depth and season in soil, and in depth on root surfaces, within a very small area of soil from one of the simpler, natural plant communities—*Calluna* heathland. The soil-plate method (Warcup, 1950) was employed for the soil studies; in use of the method, and also to some extent in presentation of results, the author has deliberately followed the procedure adopted by Warcup. In a subsequent paper, the soil-plate method will be compared with some recently described methods for isolating active mycelia from soil.

THE HABITAT

These investigations were confined to a small area (740 yd.²) of soil situated within a large expanse of *Calluna*-heath at the north-west foot of Burnt Hill on Chobham Common, Surrey. With the exception of a few scattered bushes of *Ulex minor* Roth., the vegetation consisted entirely of *Calluna vulgaris* Salisb. and had not been fired for 10 years. The *Calluna* grew in bushy habit, 12–18 in. high, and the soil surface between the stems was bare except for sparse development of *Cladonia* spp. and mosses.

The sandy soil, derived from Bagshot Beds, was podsolized and varied in reaction from pH 3.7 at the surface to pH 4.6 in the subsoil.

<i>Description of the soil profile</i>		
Horizon	Depth (in.)	
A1	2.5–4.5	Humus-rich sand with profuse development of fine, fibrous roots. Black when moist, chocolate-brown when dry
A2	12.0–19.0	Light, greyish brown sand of loose texture
B1	15.0–22.0	Firmly compacted, very dark brown
B2	17.0–28.0	Very firmly concreted, reddish-brown, gritty in texture
C	—	Orange or yellow clayey-sand

Distribution of Calluna roots. This was closely related to the soil horizons. The stout rootstock, often 1 in. thick, was situated just below and more or less parallel to the surface. Adventitious roots arose from the submerged parts of the aerial shoots. Three systems could be distinguished in the root arrangement, viz.: (i) a dense horizontal system of fine roots and hair roots (root hairs are absent from *Calluna*), profusely branched and imparting a fibrous consistency to the A1 horizon; (ii) the sparsely branched, heavily suberized, primary lateral roots which grew more or less vertically downwards through the A2 horizon; (iii) very localized yet fairly dense ramifications of very fine and hair roots in the B1 horizon. Roots were observed only rarely in the B2 and C horizons.

COLLECTION OF SOIL SAMPLES, ISOLATION AND RECORDING OF FUNGI

Profiles were exposed by digging a pit to the C horizon and soil samples were collected at every inch to a depth of 6 in. and then at every third in., generally to a depth of 30 in. At the sampling points the surface soil was

scraped away with a sterilized spatula and samples were collected by pushing sterilized glass tubes horizontally into the soil face. One set of profile samples was collected each month from October 1952 to July 1953, inclusive.

On the day of collection of the samples, soil plates (Warcup, 1950) were prepared in triplicate with each of the following media: (a) Czapek-Dox agar + 0.5% yeast extract, pH adjusted to 4.5. (b) As above but pH unadjusted and containing rose bengal (1:15,000). (c) Soil extract agar, prepared from *Calluna*-heath surface soil (40 g./l.) containing K_2HPO_4 (0.2 g.), yeast extract (0.1 g.) and glucose (1.0 g.).

The soil plates were incubated at room temperature and examined regularly during the following month. Apart from the final examination and recording, plates were opened only to excise rapidly growing fungi, or to isolate commonly occurring fungi on slopes. A fungus was given a positive record if it occurred on one or more of the three plates poured with any one of the three agar media. Thus for each soil sample a maximum of three records was possible.

FUNGI ISOLATED BY THE SOIL PLATE METHOD

In the list of fungi isolated the culture number is given where identification is incomplete or uncertain. The figure in bold type following each specific name denotes the constancy of occurrence in the ten profiles sampled.

List of fungi isolated by the soil plate method

Phycomycetes

- | | |
|--|--|
| <i>Absidia orchidis</i> (Vuill.) Hagem, 7 | <i>M. marburgensis</i> Linnem., 2 |
| <i>Haplosporangium decipiens</i> Thaxt. 3 | <i>Mucor ramannianus</i> Möll., 10 |
| <i>Mortierella isabellina</i> Oudem. 7 | <i>Piptocephalis cylindrospora</i> Bain., 2 |
| <i>M. parvispora</i> Linnem., 4 | |

Ascomycetes

- Gelasinospora cerealis* Dowd., **2**
Thielavia terricola (Gilm. & Abb.) Emm., **3**
Thielavia sp. (C25), **1**

Fungi Imperfecti

- | | |
|--|---|
| <i>Bactridiopsis</i> sp. (C21), 6 | <i>P. nigricans</i> Bain. ex Thom., 2 |
| <i>Beauveria bassiana</i> (Bals.) Vuill., 2 | <i>P. restrictum</i> Gilm. & Abb., 2 |
| <i>Cephalosporium</i> sp. (D31), 4 | <i>P. ? rolfsii</i> (E75), 1 |
| <i>Cephalosporium</i> sp. (D31a), 1 | <i>P. spinulosum</i> Thom., 9 |
| <i>?Chaetopsis</i> sp. (D34), 2 | <i>P. thomii</i> Maire, 1 |
| <i>Coniothyrium</i> sp. (C23), 2 | <i>P. thomii</i> series (E48), 1 |
| <i>Cladosporium herbarum</i> (Pers.) Link ex Fr., 8 | <i>Penicillium</i> sp. (E62), 4 |
| <i>Humicola</i> sp. (F6), 1 | <i>Penicillium</i> sp. (E67), 2 |
| <i>Oedocephalum</i> sp. (F34), 1 | <i>Phoma</i> sp. (C22), 1 |
| <i>Penicillium adametzii</i> Zal., 9 | <i>Pullularia pullulans</i> (de Bary & Löw) Berk., 8 |
| <i>P. brevi-compactum</i> Dierckx, 2 | <i>Pyrenochaeta</i> sp. (C24), 1 |
| <i>P. citreo-viride</i> Biourge, 2 | <i>Sarcinella</i> sp. (D39), 2 |
| <i>P. cyclopium</i> Westl., 4 | <i>Scopulariopsis</i> sp. (F35), 2 |
| <i>P. fellutanum</i> Biourge, 2 | <i>Trichobotrys</i> sp. (D27), 10 |
| <i>P. ?melinii</i> (E70), 1 | <i>Trichoderma viride</i> Pers. ex. Fr., 10 |
| <i>P. namyslowskii</i> Zal., 10 | <i>Tritirachium</i> sp. (F20), 1 |
| Unidentified spp.: D21, D32, D38, D44, D45, 1 | |

Non-sporing mycelia

- | | |
|--|---|
| Phycomycete mycelium (F37), 9 | Sclerotium-forming mycelium (C18), 2 |
| DS.45, 3 DS.85, 10 16 'DS' mycelia, 1 | |

Oomycetes and Basidiomycetes were not represented and only three Ascomycete species were recorded. Dominant were members of the Moniliales, of which *Penicillium* was the commonest. As in investigations of somewhat similar soils by Jefferys, Brian, Hemming & Lowe (1953) and Thornton (1956), *Fusarium* spp. were not recorded.

Of the non-sporing mycelia one (F37) was a Phycomycete, very similar in colony habit to *Mucor ramannianus*, and another (C18) was a rapidly growing, sclerotium-forming mycelium. The remaining 18 isolates were all very slowly growing, dark-coloured mycelia which formed small and generally leathery colonies; these are referred to as 'DS' forms.

No effect of the medium was noted except for *Thielavia* spp. which were recorded only on soil extract agar plates. The distribution of fungi at each of the ten levels of constancy is presented in Table 1.

Table 1. Occurrence of fungi in ten profiles

No. profiles giving occurrence ...	1	2	3	4	5	6	7	8	9	10
No. species	16	14	2	4	0	1	2	2	2	4
No. sterile mycelia	16	1	1	0	0	0	0	0	1	1

The majority of fungi were isolated from only one or two profiles and these will not be mentioned further except to note, firstly, the animal parasites *Tritirachium* sp. (F20) and *Beauveria bassiana*, and secondly, that *B. bassiana*, *Penicillium fellutanum* and *Scopulariopsis* sp. (F35) were of frequent occurrence in the profiles from which they were isolated. The few fungi of higher constancy, however, tended to dominate the soil plates and the 20 species which were isolated from more than two profiles ('constant' species) have been selected for more detailed analysis of seasonal and other effects described below.

Table 2. Frequency of occurrence of constant species in ten monthly samples

	1952			1953						
	X	XI	XII	I	II	III	IV	V	VI	VII
<i>Absidia orchidis</i>	—	7	7	7	2	—	—	2	5	—
<i>Bactridiopsis</i> sp. (C21)	—	13	9	—	5	7	10	—	14	—
<i>Cephalosporium</i> sp. (D31)	5	4	—	2	—	—	—	—	12	—
<i>Cladosporium herbarum</i>	5	7	—	5	5	—	2	2	2	14
<i>Haplosporangium decipiens</i>	5	—	—	—	—	—	—	2	7	—
<i>Mortierella isabellina</i>	17	2	—	12	17	—	2	2	36	—
<i>M. parvispora</i>	12	2	—	—	7	—	2	—	36	—
<i>Mucor ramannianus</i>	64	53	78	57	45	58	64	64	48	62
<i>Penicillium adametzi</i>	10	7	—	10	10	9	12	24	40	19
<i>P. cyclopium</i>	2	2	—	7	2	—	—	—	—	—
<i>P. namyslowskii</i>	10	20	18	7	31	11	2	17	17	17
<i>P. spinulosum</i>	7	13	24	14	12	7	7	—	7	10
<i>Penicillium</i> sp. (E62)	5	4	—	2	—	7	—	—	—	—
<i>Pullularia pullulans</i>	14	2	7	5	2	7	—	—	17	12
<i>Thielavia terricola</i>	5	—	—	—	—	2	—	—	—	2
<i>Trichobotrys</i> sp. (D27)	24	31	47	36	45	22	19	38	14	17
<i>Trichoderma viride</i>	24	16	16	14	36	20	26	7	12	14
Phycomycete mycelium (F37)	—	9	11	28	5	16	35	5	19	10
Sterile mycelium (DS45)	2	—	—	—	—	7	—	2	—	—
Sterile mycelium (DS85)	—	7	27	14	17	2	5	5	5	5

EFFECT OF SEASON ON THE ISOLATION OF FUNGI

The occurrence of the 'constant' species over 10 months (expressed as percentage total number of records possible) is presented in Table 2.

There was no evidence that the total incidence of species, or the incidence of individual species, varied with season. *Penicillium adametzi* appeared more frequently in the summer and sterile mycelium DS85 in winter, but it is clear from the non-seasonal variation in other fungi, e.g. phycomycete mycelium F37, that this could be a chance distribution. It must be concluded from these results, therefore, that if seasonal fluctuation did in fact occur, it was not demonstrated by the soil plate method (cf. Warcup, 1951).

VERTICAL DISTRIBUTION OF FUNGI

The numbers of sporing species isolated decreased with depth (Fig. 1). Only a few isolates were obtained from the lower illuviated horizons and parent subsoils and plates prepared from these levels were frequently sterile. The 'DS' sterile mycelial forms showed a reverse of this distribution and accounted for more than half the total number of fungi isolated from the B₁, B₂ and C horizons.

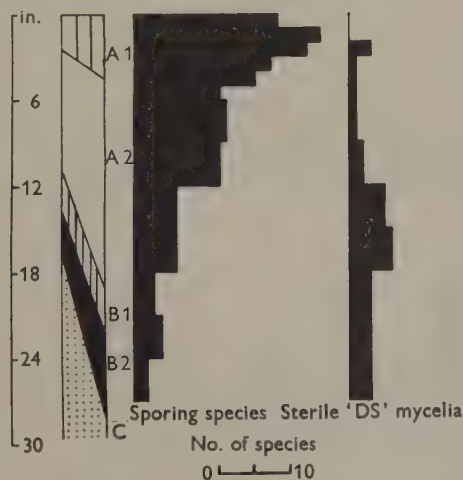


Fig. 1. Vertical distribution of fungi in relation to profile structure.

In Fig. 2, the total numbers of records for Ascomycetes and species of *Mortierella* and *Penicillium* are plotted against the profile horizons and it will be seen that they were largely confined to the A₁ horizon, this being particularly marked with the *Penicillia*.

In general the frequency of occurrence of individual species also decreased with depth. Exceptions to this, however, were *M. ramannianus*, *Trichobotrys* sp. (D27) and the sterile, phycomycete mycelium (F37), which were common in the A₂ and B₁ horizons, and the sterile mycelium (DS85), which was most frequently encountered in the B₂ and C horizons (Fig. 3).

Indeed the high frequency of a small number of species in the A2 and B1 horizons was perhaps the most striking feature of the investigation. On soil plates prepared from these horizons, colonies of *Mucor ramannianus*, *Trichobotrys* sp. (D27) and phycomycete mycelium (F37) developed in abundance and, apart from the occasional incidence of *Trichoderma viride* and *Penicillium namslowskii*, were often the only fungi present.

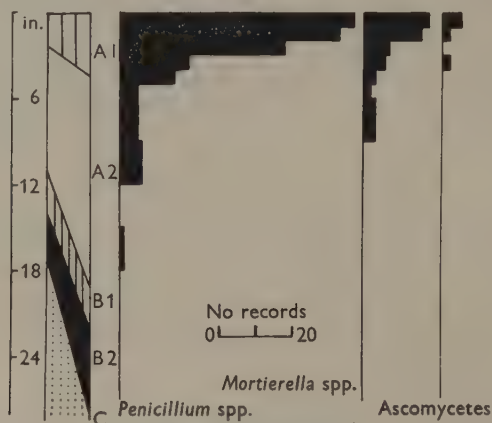


Fig. 2. Occurrence of three groups of fungi in relation to profile structure.

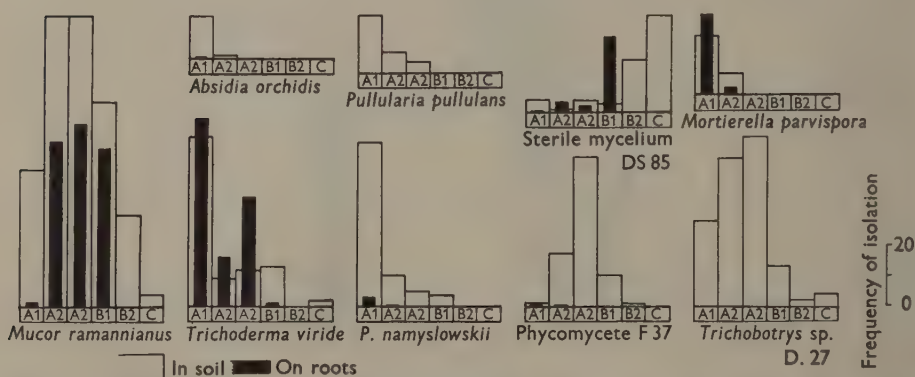


Fig. 3. Occurrence of fungi in soil and on root surfaces. *N.B.* Roots were not sampled from the B2 and C horizons.

Although distribution in the lower horizons was not generally related to absolute depth within them, there did exist a marked vertical zonation within the A1 horizon and also, to a lesser extent, within the more superficial levels of the A2 horizon (Table 3). These intra- and inter-horizon distributions were often closely related; thus species occurring only in the A1 horizon were isolated infrequently below the first inch. *Trichoderma viride* occurred on every soil plate prepared from the first inch but with decreasing frequency from deeper levels. Both *Mucor ramannianus* and *Trichobotrys* sp. (D27), of very frequent occurrence in the A2 horizon,

Table 3. Frequencies of occurrence of fungi in three sampling levels of the A₁ horizon, and distribution in five soil horizons

	A ₁ Horizon (in.)			Horizon					
	1	2	3	A ₁	A ₂	A ₂	B ₁	B ₂	C
<i>Mortierella marburgensis</i>	10	—	—	+	—	—	—	—	—
<i>Gelasinospora cerealis</i>	7	—	—	+	—	—	—	—	—
Sterile mycelium (C ₁₈)	7	—	—	+	—	—	—	—	—
<i>Haplosporangium decipiens</i>	13	10	—	+	—	—	—	—	—
<i>Thielavia terricola</i>	10	7	—	+	—	—	—	—	—
<i>Absidia orchidis</i>	50	7	—	+	—	—	—	—	—
<i>Mortierella parvispora</i>	17	10	10	+	+	—	—	—	—
<i>Penicillium spinulosum</i>	63	37	20	+	+	+	—	—	—
<i>Pullularia pullulans</i>	30	20	7	+	+	+	—	—	—
<i>Penicillium adametzi</i>	60	67	40	+	+	+	—	—	—
<i>Mortierella isabellina</i>	27	47	20	+	+	+	—	—	—
<i>Bactridiopsis</i> sp. (C ₂₁)	10	17	10	+	+	+	—	—	—
<i>Trichellium</i> sp. (E ₆₂)	7	10	3	+	+	+	—	—	—
<i>P. namyslowskii</i>	73	50	30	+	+	+	+	—	—
<i>Trichoderma viride</i>	100	57	27	+	+	+	+	—	+
<i>Mucor ramannianus</i>	7	60	70	+	+	+	+	+	+
<i>Trichobotrys</i> sp. (D ₂₇)	—	20	43	+	+	+	+	+	+
Sterile mycelium (D _{S.85})	—	—	17	+	+	+	+	+	+

were rarely isolated from surface soil. The vertical distributions could, with few exceptions, be related to the growth rates of fungi on soil plates and it is possible that the competition of rapidly growing species greatly influenced the isolation of those with lower growth rates. The increased incidence with depth of the sterile, dark mycelial forms may well have been determined by the reduced or negligible competition on plates prepared from deeper soils.

OCCURRENCE OF FUNGI ON ROOT SURFACES

Collection of roots. Samples of *Calluna* roots were collected from the A₁, upper and lower levels of the A₂ and the B₁ horizons, and classified according to their thickness. The range of sizes and numbers of roots sampled from four soil levels through three horizons are recorded in Table 4. In some horizons certain types of roots were too sparse for adequate sampling.

Roots were collected directly from the soil except for the compacted B₁ horizon, from which roots were obtained by soaking, washing and sieving fairly large quantities of soil in the laboratory.

Table 4. Numbers of root fragments sampled

Root diam. (mm.)	Soil levels			
	A ₁	A ₂ (U)	A ₂ (L)	B ₁
2.0	100	100	—	—
1.0-2.0	100	100	100	—
0.5-1.0	100	100	100	—
0.5	200	100	100	200
Total	500	400	300	200

Method of isolation. Roots were washed in running tap water to remove all soil particles, cut into short lengths (*c.* 2 mm.) and then washed for 6 min. in each of five changes of sterile water, using a washing device of the type described by Simmonds (1930). Washed root fragments were blotted dry on sterile filter-paper and then transferred to plates of Czapek-Dox agar (Warcup, 1950). All roots were collected during the period October 1953 to January 1954 and all were plated on the day of collection.

Results of root surface isolations

There was no evidence that distribution of fungi was determined by root type. The fungi isolated are therefore shown with reference only to their frequency in different horizons (Table 5). Fungi not isolated by the soil plate method are indicated by an asterisk.

Table 5. *Frequency of occurrence of fungi on root surfaces*

	Horizon			
	A 1	A 2(U)	A 2(L)	B 1
<i>Absidia orchidis</i>	0.6	—	—	—
<i>Mortierella isabellina</i>	0.2	—	—	—
<i>M. marburgensis</i>	7.6	—	—	—
<i>M. parvispora</i>	25.8	2.0	—	—
* <i>M. zychae</i>	0.4	—	—	—
* <i>Mucor hiemalis</i>	0.2	—	—	—
* <i>M. piriformis</i>	1.6	—	—	—
<i>M. ramannianus</i>	0.8	53.7	60.3	52.0
Sterile Phycomycete (F 37)	0.4	0.5	—	—
* <i>Botrytis cinerea</i>	0.6	—	—	—
<i>Cladosporium herbarum</i>	0.4	0.5	—	—
* <i>Coniothyrium</i> sp. (C 33)	—	3.0	—	—
* <i>Coniothyrium</i> sp. (C 36)	—	—	2.7	—
* <i>Coniothyrium</i> sp. (C 37)	—	0.3	—	—
* <i>Gliobotrys</i> sp. (D 46)	—	0.3	12.0	1.5
<i>Penicillium adametzi</i>	0.2	1.5	—	—
<i>P. citreoviride</i>	—	1.3	—	—
* <i>P. lanosum</i>	0.8	—	—	0.5
<i>P. namyslowskii</i>	2.8	0.4	—	—
<i>P. raistrickii</i> series (E 72)	—	0.3	—	—
<i>P. spinulosum</i>	9.0	0.4	0.7	—
* <i>P. terlikowskii</i>	—	—	6.3	—
<i>P. thomii</i>	0.2	—	—	—
<i>P. thomii</i> series (E 48)	0.4	—	—	—
* <i>P. viridicatum</i>	30.8	2.3	20.3	—
<i>Penicillium</i> sp. (E 62)	—	—	0.3	—
* <i>Penicillium</i> sp. (E 74)	0.6	0.3	—	—
* <i>Phoma</i> sp. (C 2)	—	6.0	—	—
<i>Phoma</i> sp. (C 22)	0.2	—	—	—
* <i>Torula</i> sp. (D 48)	—	—	1.3	7.5
<i>Trichoderma viride</i>	62.4	16.5	37.3	1.0
Sterile mycelium (DS.85)	0.4	3.3	2.0	25.0
*9 Sterile mycelia (DS)	1.0	4.8	—	11.5

Ascomycetes, Basidiomycetes and Oomycetes were not isolated by this method. Fourteen of the 41 species, including four members of the Sphaeropsidales, had not been isolated directly from soil. Only two of these, however, *Penicillium viridicatum* and *Gliobotrys* sp. (D 46), were frequent.

The distribution of fungi on roots generally followed closely that in soil. Thus members of the Mucorales were confined to roots from the A1 horizon, with the exception of *Mucor ramannianus* which occurred frequently only on roots from the A2 and B1. The 'DS' mycelial forms also were isolated most frequently from roots of deeper soil horizons. Instances of discontinuity in the vertical distribution of certain species (*Penicillium viridicatum* and *Trichoderma viride*) is probably due to localization of sampling but may indicate the occurrence of local abundance or 'flushes' of these species on root surfaces. In fig. 3, the distribution of a few species on root surfaces and in soil are compared diagrammatically.

DISCUSSION

Since the incidence of species was apparently unrelated to season, it was possible to compare directly the ten monthly profile samples. In evaluating the results, constancy rather than frequency of occurrence was employed initially, as this was thought further to diminish the selective advantage of high sporing capacity. Generally, however, in this soil, constancy and frequency were fairly closely related.

The surface flora of *Calluna* roots was related more to soil horizon than to root type. Thus the vertical distributions were more or less the same for fungi in soil and on roots; certain species, however, in particular *Trichoderma viride* and *Mortierella* spp., were more prevalent on roots as judged by the relative frequencies of isolation.

The majority of sporing species occurring on soil plates were of low constancy and frequency and were isolated largely from the surface (A1) horizon. In the subsurface horizons the fungal community was more stable, being represented by few species of both high constancy and high frequency. In general the most rapidly growing fungi occurred in the superficial levels of the surface horizon and it is perhaps of some ecological significance that these species, because of their rapid growth rate (which in this sense must also include the 'lag phase' described by Park, 1954) may gain considerable advantage in the colonization of organic matter freshly deposited at the surface. Such an advantage is given added significance in consideration of the poor quality and relative sparsity of *Calluna* debris. This latter feature of the habitat may explain, in part, the divergence between the results in Table 3 and those of Warcup (1951), who concluded that the vertical distribution of fungi in grassland soils was correlated with the soil horizon rather than with depth in inches.

The vertical distribution patterns demonstrated for such fungi as *Trichoderma viride*, members of the Mucorales and Ascomycetes, and *Penicillium* spp., are probably true representations; those of the more slowly growing fungi (e.g. *Mucor ramannianus*, the 'DS' mycelia and *Trichobotrys* sp. (D27)), however, may have been affected by competition on isolation plates. Nevertheless, these latter appear to be the dominant representatives of a very stable flora of the subsurface horizons. Such relatively small, subsurface populations dominated by *Mucor ramannianus* have also been recorded from *Calluna*-heathland soils by Jefferys *et al.* (1953), Thornton (1956) and McLennan & Ducker (1954). In view of

the demonstration by Newman & Norman (1941) that increase with depth in stability of nutrient and environmental conditions is shown most clearly where profile development is sharp, this *M. ramannianus* association might be regarded as a community in 'approximate equilibrium with all the conditions in which it exists' (Tansley, 1949). Although the ultimate microhabitats, as stated by Stanier (1953), may be omnipresent, the conditions of the macrohabitat must govern in varying degrees the species colonizing them (Park, 1955); these conditions would generally be expected to be imposed more rigorously at the lower soil levels (Newman & Norman, 1941, 1943) and particularly at these levels in podsolized soils beneath such an (anthropogenic) climax community as *Callunetum*.

This investigation was carried out initially during the tenure of a Postgraduate Studentship at Royal Holloway College, University of London, and later on a Nature Conservancy Research Studentship. I wish to express my gratitude to Prof. F. W. Jane, D.Sc., and Dr M. P. Topping for their help and guidance. I am indebted to Mrs M. Turner, Dr M. B. Ellis, Dr E. W. Mason and Mr G. Smith, who identified representative cultures of fungi. The work described forms part of a thesis presented for the degree of Doctor of Philosophy in the University of London, 1954.

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(Accepted for publication 15 November 1958)

STUDIES OF FUNGI IN A *CALLUNA*-HEATHLAND SOIL

II. BY THE COMPLEMENTARY USE OF SEVERAL ISOLATION METHODS

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(With 2 Text-figures)

The soil plate, immersion tube and slide-trap methods were used in an investigation of the fungus flora of a small area of *Calluna*-heathland surface soil. A marked seasonal variation in the activity of *Trichoderma viride* was demonstrated by the two latter ('direct') methods. The isolation method and medium, time, position and depth of sampling, and interactions between these factors, differentially affected the results. In general, the soil-plate method favoured the isolation of heavily sporing fungi, particularly *Penicillium* spp., and the direct methods emphasized the importance, perhaps unduly, of *Mortierella* spp. and other very rapidly growing fungi. It is suggested that a physical 'rhizosphere' effect might have been produced by the immersion in soil of solid objects, which stimulated the growth of certain fungi. Competition between species was a major factor in determining the results obtained by all methods.

These results were augmented by studies of two specific habitats and by direct observation. An attempt was made to outline certain mycelial space relationships by an analysis combining all methods of study. The direct isolation methods appeared, in part, to be selective for translocating types of mycelia. Even with the use of these several methods, it was apparent that a proportion of the fungus flora, which consisted largely of humus-inhabiting fungi, was unavailable for study.

INTRODUCTION

It is generally accepted that all methods of isolating fungi from soil are selective and that a single non-selective method is unlikely ever to be elaborated (Garrett, 1951). Harley & Waid (1955) consider that such a non-selective method would yield information of less value than others by which selection is consciously employed to describe the pattern arrangement of a soil flora: at the same time they emphasize the importance of stating strictly the limits of accuracy and sensitivity of any method used. The degree of accuracy outlined for any one method in any particular investigation is, however, relative only to other methods simultaneously employed; as the number of complementary methods is increased so the degree of accuracy can be more precisely defined. That information of great value can be obtained by the complementary use of a number of methods has been demonstrated by the recent work of Chesters (1948), Warcup (1957) and Brown (1958).

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In the present investigation, three recently described isolation methods, augmented by isolations from two specific habitats and by direct observations, have been employed to study the microfungi in the surface horizon of a *Calluna*-heathland soil. An attempt has been made to state the most important factors affecting the results and also to derive information concerning the growth habits of a few fungi.

MATERIALS AND METHODS

The soil

The general characteristics of the podsolized, *Calluna*-heathland soil studied have been described previously (Sewell, 1959). The present investigations were confined to the surface, A1 horizon, which varied in depth from 2.5 to 4.5 in. This soil was markedly acid in reaction (pH 3.6–3.8) and possessed a high moisture-holding capacity (71 % air-dry weight). Fine, fibrous roots of *Calluna* were densely developed, particularly in the most superficial levels (0–1.5 in.).

The soil plate (Warcup, 1950) and root surface isolation methods

The isolation methods used for soil and roots, and the results obtained, have been described previously (Sewell, 1959). The results quoted for purposes of the present discussion are of isolations from soil at 1, 2 and 3 in. and from roots of the A1 horizon.

The immersion tube method (Chesters, 1940, 1948)

Immersion tubes were prepared and used in a manner similar to that described by Chesters, except that the agar core, sampled from the tube, was cut before plating into four pieces only. In routine isolations the following media were used: (a) heathland-soil-extract agar (SEA) (Sewell, 1959); (b) Czapek-Dox agar + 0.5 % yeast extract, pH 4.5 (CDY); (c) 'Difco' cornmeal agar (CMA); (d) plain water agar with 3 % agar (PWA).

Thirty-six immersion tubes, nine tubes for each of the four media, were buried in the soil every month from October 1952 to January 1954 inclusive, with the exceptions of November 1952 and July and December 1953. Spare immersion tubes were removed after varying periods of incubation in the soil and only when mycelia were observed in these were the test batches collected. Even so, during winter months, when 20 days or more incubation was necessary, several tubes often remained uninvaded. The total number of immersion tubes successfully employed was 386.

In order to determine whether fungi were generally or locally active, the tubes were distributed in compact groups over the sampling area, each group containing tubes with the four media in equal proportion.

The slide-trap method

The design, preparation and use of the modified slide-trap have been described elsewhere (Sewell, 1956). As it was developed during these investigations it was put into use only during the latter part of the period,

ten slide-traps being used in August and ten in November 1953, and 18, 15 and 21 in January, February and March 1954, respectively. Plain water agar (PWA) was used throughout.

Isolation of fungi from rabbit dung

During July and August 1953, pellets of rabbit dung were collected from well-distributed areas over the Burnt Hill site. Very small quantities of material from the centre of each pellet were sampled by the soil-plate method (Warcup, 1950).

The Rossi-Cholodny slide method (Cholodny, 1930)

Clean microscope slides were buried in soil for 7 or 14 days. After removal they were dried at room temperature and fixed by gentle heat. The largest soil particles were removed by agitation in water and the slides were then stained for 1 hr. in phenolic aniline blue (Jones & Mollison, 1948). A total of fifty slides was immersed in the surface soils during the period September–November 1953.

FACTORS AFFECTING THE ISOLATION OF FUNGI

The fungi isolated by each method, together with their frequencies of isolation (represented by a percentage of the maximum possible number of records), are recorded in Table 1. With few exceptions each fungus was isolated with notably greater frequency by one particular method or, in some extreme cases, with high frequency by one method alone (e.g. *Mucor ramannianus*, basidiomycete mycelium C34, *Penicillium adametzi*). Some of the factors which appeared to be most important in differentially affecting the results are considered below.

The isolation medium

The media used in immersion tubes markedly affected the fungi isolated (Table 2). While *Trichoderma viride* and the sterile mycelium C18 were frequent in all four media, Ascomycetes and *Mucor* spp. were isolated infrequently on PWA—a medium which favoured the isolation of *Mortierella zychnae*. A similar effect of medium (and also a more even isolation of fungi using SEA) has been noted by Chesters (1948).

This effect of medium was not constant throughout the range of methods. Thus, no differential effect was noted for the three media used in soil plates (CDY, SEA, CDY + rose bengal), except possibly for *Thielavia* spp. which were recorded only on SEA plates. The importance of the interaction between medium and method was demonstrated by the fact that whereas *Mortierella marburgensis* and all *Mucor* spp. were isolated frequently by CDY in immersion tubes, they were isolated very infrequently by the same medium in soil plates.

The results for immersion tube isolations using PWA (Table 2) generally agree closely with those obtained with this medium in slide-traps (Table 1). Whilst it is probable that the negligible isolation of Ascomycetes and *Mucor* spp. by the latter method was due to the use of PWA only

Table 1. *Frequency of occurrence of fungi*

	Method of isolation				
	Immer- sion tube	Slide trap	Soil plate	Root plate	Dung plate
<i>Absidia orchidis</i> (Vuill.) Hagem	1	1	16	1	—
<i>Haplosporangium decipiens</i> Thaxt.	2	15	6	—	—
<i>Mortierella bainieri</i> Cost.	2	4	—	—	—
<i>M. hygrophila</i> Linnem.	1	1	—	—	—
<i>M. isabellina</i> Oudem.	—	7	28	1	—
<i>M. marburgensis</i> Linnem.	9	8	3	—	—
<i>M. parvispora</i> Linnem.	3	22	14	26	—
<i>M. pulchella</i> Linnem.	1	1	—	—	—
<i>M. zychnae</i> Linnem.	25	39	—	1	—
<i>Mucor hiemalis</i> Wehmer	9	1	—	1	6
<i>M. ?mucedo</i> (Linne) Bref. (A39)	1	—	—	—	2
<i>M. piriformis</i> Fisch.	2	1	—	1	11
<i>M. ramannianus</i> Möll.	*	*	43	1	2
<i>M. saturninus</i> Hagem	2	—	—	—	4
<i>Pilaira anomala</i> (Ces.) Schroet.	—	—	—	—	11
<i>Piptocephalis</i> sp. (A23)	—	—	2	—	—
<i>Gelasinospora cerealis</i> Dowd.	3	—	2	—	19
<i>G. retispora</i> Cain.	2	—	—	—	17
<i>Sordaria destruens</i> (Shear) Hawk.	1	—	—	—	4
<i>S. humana</i> (Fuck.) Wint.	1	—	—	—	6
<i>Sporormia intermedia</i> Auwers.	—	—	—	—	21
<i>Thielavia terricola</i> (Gilm. & Abb.) Emm.	—	—	4	—	—
<i>Thielavia</i> sp. (C25)	—	—	1	—	—
<i>Bactridiopsis</i> sp. (C21)	7	3	12	—	—
<i>Beauveria bassiana</i> (Bals.) Vuill.	—	*	5	—	—
<i>Botrytis cinerea</i> Fr.	8	4	—	1	4
<i>Cephalosporium</i> sp. (D31)	—	—	2	—	—
<i>Cladosporium herbarum</i> (Pers.) Link ex Fr.	—	—	10	1	11
<i>Coniothyrium</i> sp. (C23)	—	4	3	—	—
<i>Coniothyrium</i> sp. (C35)	—	1	—	—	—
<i>Coniothyrium</i> sp. (C38)	—	—	—	—	11
<i>Dicoccum asperum</i> Corda	1	—	—	—	—
<i>Epicoccum</i> sp. (D35)	4	—	—	—	—
<i>Humicola</i> sp. (F6)	1	1	1	—	—
<i>Penicillium adametzii</i> Zal.	—	—	48	1	—
<i>P. citreo-viride</i> Biourge	—	—	3	—	—
<i>P. cyclopium</i> Westl.	—	—	5	—	—
<i>P. fellutanum</i> Biourge	—	—	13	—	—
<i>P. namyslowskii</i> Zal.	*	18	51	3	11
<i>P. spinulosum</i> Thom	—	—	36	9	9
<i>P. thomii</i> Maire.	—	—	2	1	—
<i>P. viridicatum</i> Westl.	—	—	—	31	9
<i>Penicillium</i> sp. (E62)	—	—	6	—	—
6 <i>Penicillium</i> spp.	—	—	2	—	—
3 <i>Penicillium</i> spp.	—	—	—	1	—
<i>Phoma</i> sp. (C22)	4	22	1	1	—
<i>Pullularia pullulans</i> (de Bary & Löw) Berkh.	—	—	18	—	—
<i>Scopulariopsis</i> sp. (F35)	—	—	3	—	—
<i>Trichobotrys</i> sp. (D27)	—	—	23	—	—
<i>Trichoderma viride</i> Pers. ex Fr.	58	61	54	62	45
Moniliaceae 3 unidentified spp.	—	—	2	—	—
Non-sporing mycelia					
Phycomycete mycelium (F37)	*	—	1	—	—
Basidiomycete mycelium (C34)	1	30	—	—	—
Sclerotial mycelium (C18)	23	30	2	—	—
Mycelium (DS.85)	—	—	5	1	—
Mycelium (HS.21)	—	1	—	—	—
2 mycelia (DS forms)	—	—	2	—	—
9 mycelia (DS forms)	—	—	—	1	—
Total number of species	25	22	43	29	18

* Isolated, but not in routine investigations.

(Brown, 1957), the importance of the medium-method interaction was again demonstrated by the high frequency of isolation of the sterile basidiomycete (C34) in slide-traps and its virtual absence from immersion tubes (Table 2).

Table 2. *Frequency of isolation of fungi in four media by the immersion tube method*

	PWA	SEA	CDY	CMA
<i>Mortierella zychnae</i>	38.5	18.3	13.5	21.0
<i>M. marburgensis</i>	8.4	3.7	16.8	8.6
Other <i>Mortierella</i> spp.	6.3	6.1	8.4	2.4
<i>Mucor</i> spp. (total)	3.5	14.7	30.1	11.0
<i>Mucor hiemalis</i>	1.4	9.7	20.5	8.6
Ascomycetes	3.5	8.5	7.2	7.2
Fungi Imperfecti (total)	68.1	80.5	82.9	72.8
<i>Trichoderma viride</i>	59.0	57.3	54.0	56.8
Sterile mycelium (C18)	20.0	25.5	15.7	28.5

Position of sampling

The absence of any evidence for seasonal variation of common fungi in soil-plate isolations (Warcup, 1951) and the high proportion of fungi of low constancy of occurrence (Sewell, 1959) suggest that these latter species were localized in spatial rather than in seasonal distribution. This tentative conclusion is supported by Warcup's (1951) statement that variation in fungal populations from soil samples collected a few yards apart on the same date was as great as that recorded for samples taken over a period of time. It seems unlikely, however, that many fungi playing *active* and *important* roles (i.e. the characteristic species) in soil microbiological processes in a uniform, undisturbed habitat (as the *Calluna*-heathland investigated) would show a limited distribution and, therefore, low constancy of occurrence (cf. Thornton, 1956). The complementary use of the 'constancy' concept with the soil-plate method, therefore, should probably be considered obligatory if this method is used in studies of natural, stable habitats for the purposes of classifying soil fungal populations, or of determining 'soil fungal patterns' (Thornton, 1956).

Evidence to support the view that active, characteristic fungal species are unlikely to show localized distribution was obtained by placing immersion tubes, each month, in well-distributed groups within the sampling area. Rarely was a fungus isolated from tubes within one group only; even those isolated but twice or thrice in a monthly sampling occurred in tubes from two or three groups. Exceptions to this widespread distribution occurred infrequently with Ascomycetes and *Mucor* spp.

Time of sampling

Marked seasonal variation in the isolation of nearly all fungi was demonstrated by the immersion tube method and also, in the more limited samplings, by the slide-trap method. The monthly isolation results obtained by these two methods are presented in Fig. 1. It is interesting to compare them with those obtained by the soil plate method (Sewell, 1959) where no variation was demonstrated for any fungus.

The occurrence of *Trichoderma viride* was closely related to temperature and, in the summer months, it dominated the isolations by direct methods (i.e. immersion tube and slide-trap). All other fungi, with exception of the sterile mycelium (C18), were isolated most frequently during periods of low temperature: it is probable, however, that this was merely a reflexion of the reduced competition from *T. viride*. This conclusion is supported by direct observations, for *Mortierella* spp. were observed to grow vigorously over Rossi-Cholodny slides during periods when they were rarely isolated in the immersion tubes. Nicot & Chevaugéon (1949), commenting upon the suppression of other fungi by *T. viride*, concluded that the vigorous growth and inhibitory properties of this fungus were responsible for its isolation alone in immersion tubes. The infrequent isolation of *T. viride* by immersion tubes from soils in which Chesters (1948) reported the fungus to be common, can perhaps be attributed to the influence of season on its activity.

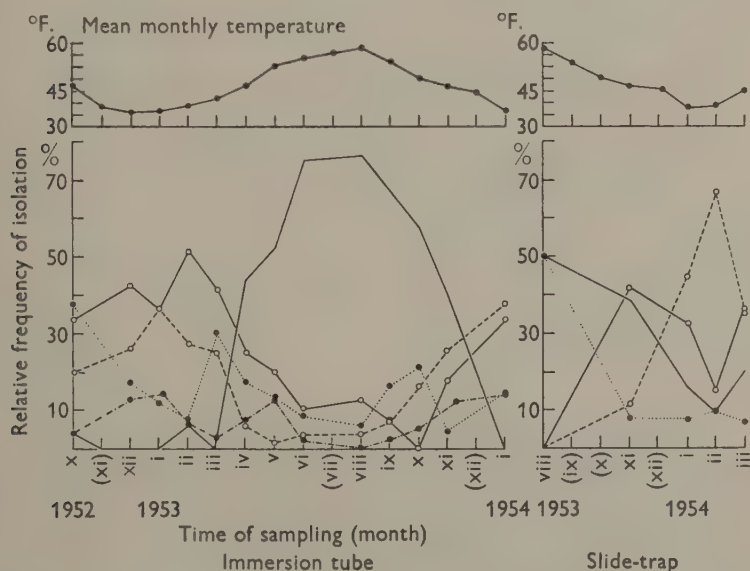


Fig. 1. Effect of time of sampling on the isolation of fungi by immersion tubes and slide-traps (no isolations were attempted during the months given in parentheses). —, *Trichoderma viride*; ○—○, *Mortierella* spp.; ●—●, *Mucor* spp.; ●·····●, sterile mycelium C18; ○—○, other fungi.

It is evident that the soil-plate method fails to differentiate between the active and relatively, or completely, dormant phases in the growth cycles of soil fungi. Its comparison, therefore, with direct isolation methods is of value only when the latter methods are employed throughout a seasonal cycle. To obtain the greatest possible information from two methods such as the soil plate and immersion tube, isolations must be made in both space (position) and in time.

Reference to isolations by immersion tube and slide-trap at similar times of the year (Fig. 1) demonstrate that, even where two direct isolation methods are used, slight differences may be exhibited in seasonal distribution patterns.

Depth of sampling

The marked effect of depth of sampling on isolations of fungi within the top 3 in. of soil by the soil-plate method has been recorded (Sewell, 1959, Table 3). In order to determine whether a similar 'layering' or zonation of species influenced isolations with immersion tubes, 'one-sided' tubes were prepared in which three capillary inlets were placed in line with the longitudinal axis of the tube. This design enabled tubes to be buried horizontally in a soil profile with all three inlets at a specific depth and facing downwards, thus precluding the possibility of contamination by falling particles. The results of two experiments using such tubes, with PWA as the isolation medium, are presented in Table 3 (isolations from the A2 horizon are included as they provide an interesting comparison with isolations from this horizon reported earlier (Sewell, 1959)).

Table 3. Results of isolations using 'one-sided' immersion tubes

	Sampling depth (in.)									
	Expt. 1		Expt. 2							
	1	3	2	3	4	5	7	9	11	13
<i>Mortierella zychnae</i>	2	—	—	—	—	—	—	—	—	—
<i>Mucor ramannianus</i>	—	—	—	4	5	5	3	5	5	5
<i>Penicillium namyslowskii</i>	—	—	—	1	—	—	—	—	—	—
Phycomycete (F37)	—	—	—	—	—	—	—	—	1	—
<i>Trichoderma viride</i>	10	—	5	1	—	—	1	—	—	—
No. tubes immersed	12	10	5	5	5	5	5	5	5	5
Soil horizon	A1				A2					

Both experiments were carried out at a time when *T. viride* was being isolated most frequently in vertically immersed tubes. Tubes of *Experiment 1* were collected when mycelia first appeared in a duplicate set of 'sample' tubes; only those in the uppermost inch of soil were colonized. Thus, although normal, vertically immersed tubes theoretically sample soil over the range of depth of the capillary inlets (i.e. 3 in., allowing for slight variation in depth of immersion), invasion may occur most rapidly through the uppermost inlets, possibly resulting in colonization of the medium before fungi growing at lower levels enter the tube. Competition not only between species entering at capillary inlets (Chesters, 1948; Nicot & Chevaugnon, 1949), but also between those already established and those attempting to enter elsewhere, may be of considerable importance.

The apparent restriction of mycelial activity of *T. viride* to the most superficial soil layer was confirmed in *Experiment 2*, and supports the results obtained by soil plate isolations (Sewell, 1959, Table 3). The isolations of *Mucor ramannianus* and *Penicillium namyslowskii* at 3 in. depth again suggests (in view of their absence from normally immersed tubes) that

rapid mycelial penetration through the uppermost inlets, followed by vigorous colonization of the agar medium, may preclude the isolation, in normal tubes, of other, more slowly growing, mycelia, penetrating through lower inlets.

COMPOSITION OF THE FUNGUS FLORA AS DEMONSTRATED BY THREE METHODS OF ISOLATION

The overall composition of the fungus flora, as demonstrated by the soil plate, immersion tube and slide-trap methods, respectively, is represented diagrammatically in Fig. 2, where the group 'sterile mycelia' includes phycomycete and basidiomycete mycelia. These figures demonstrate the dominance of Mucorales (particularly *Mortierella* spp.) and Fungi Imperfecti (with exception of the *Penicillia*) in isolations by the direct methods, and dominance of Fungi Imperfecti (particularly *Penicillia*) in soil plate isolations.

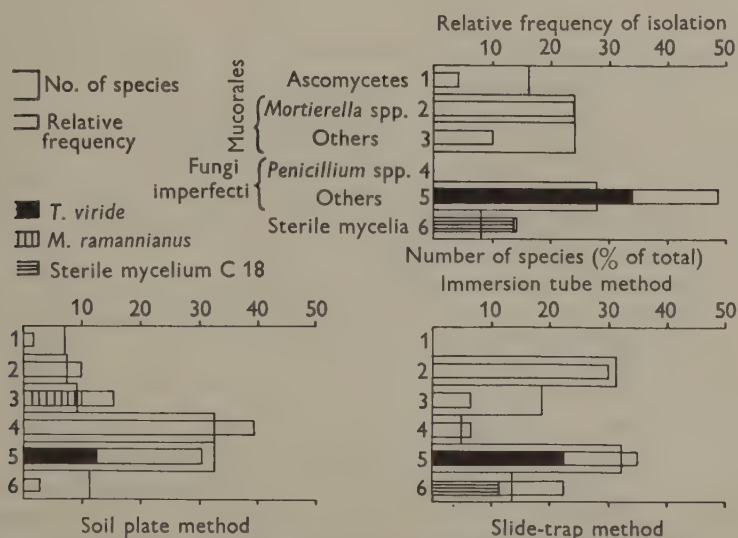


Fig. 2. Composition of the fungus flora as demonstrated by three methods of isolation.

Ascomycetes were poorly represented and, although a number of species were isolated in immersion tubes, they occurred infrequently. *Gelasinospora retispora* does not appear previously to have been recorded from soil.

*Mortierella*s were well represented, both in number of species and frequency, by the direct methods, whereas on soil plates few species occurred yet with relatively high frequency. The results from both methods were considerably influenced by the occurrence of *Mortierella zychae* and *M. isabellina*, respectively; the former possesses a high growth rate and relatively low sporing capacity and the latter the reverse of these characteristics (see Table 2).

Similarly, other species of Mucorales were well represented by direct methods but poorly by soil plates: the single exception, *M. ramannianus*,

was again a species not typical of the genus—possessing a very high sporing capacity and relatively low growth rate. Isolation of this fungus by direct methods was evidently influenced by the competition of faster growing species, and the difference between results from different methods was, in this case, largely associated with depth of sampling.

Penicillium spp., which dominated soil plate isolations, were virtually unrepresented by direct methods. It is noteworthy that the commonest species on soil plates, *P. namyslowskii*, was isolated alone, but not infrequently, in slide-traps and also in immersion tubes, in the absence of competition from more vigorous fungi (Table 3).

Many other imperfect fungi were isolated by all methods, but, with the exception of *Trichoderma viride* and sterile mycelium C18, they were infrequent.

With few exceptions, the direct methods tended to select rapidly growing fungi, and the soil plate those of medium to high growth rate and high sporing capacity.

DIRECT OBSERVATION BY THE ROSSI-CHOLODNY SLIDE METHOD

Occurrence of hyaline or pale mycelia

Slides prepared from the surface soil layers (0–2 in.) were richly covered with hyaline, generally septate, mycelia which varied greatly in intensity of staining. Often the staining was deepest near the hyphal tips (Jones & Mollison, 1948), but spore-bearing structures and ‘foot-cells’ of *Mortierella* spp. were invariably deeply stained.

Although the majority of mycelia were not sporulating some, particularly *Mortierella* spp., were: *M. marburgensis* was common and other sporing mycelia were attributed to *M. parvispora* and *M. zychae*, but five species commonly observed on slides could not be related to any of those isolated and recorded in this investigation.

The commonest sporing hyphomycetous mycelia were cephalosporic in habit and while these may, in fact, have been *Cephalosporium* spp., fungi of other genera have been observed to adopt such a sporing habit in soil (Kubiena, 1938). In three of the four preparations with sporing *Penicillia*, they were associated with a dead nematode and insect remains, and on the fourth the mycelium was intimately associated with a fragment of unidentified organic material. All animal remains were heavily colonized by fungal mycelia. Sporing hyphae of *Acrostalagmus*(?) *obovatus* were observed growing from a nematode: this observation adds to the limited evidence for nematode predation by fungi under natural conditions (Capstick, Twinn & Waid, 1957).

On slides prepared from soils of 0–2 in. depth, little was observed of the other, commonly isolated fungi. Broad coenocytic mycelia of the mucoraceous type were rare and no (sporulating) mycelium of *Trichoderma viride* was seen with certainty. The great amount of hyaline mycelium observed was sterile and could not be identified, and so the failure to identify some commonly isolated fungi was not surprising.

Slides immersed at 3 in. were almost barren of hyaline mycelia and the only sporulating form encountered was attributed to *Mucor ramannianus*.

Humus pellets, abundant nearer the surface, were not commonly observed at 3 in., but a delicate, fur-like humic substance coated many sand grains. Mycelium of the *M. ramannianus* type, frequently bearing globose chlamydospores, was observed occasionally to develop from these coated grains. The reduction of mycelial development at 3 in. (and below) was considerably greater than was expected from soil-plate isolations.

Occurrence of dark-coloured mycelia

Dark, septate mycelia were very common on soil slides: they differed from hyaline mycelia in habit, form and distribution. Sporulation was rare and the only identifiable mycelium (from 1 in.) was of *Trichobotrys* sp. (D 27). Dark hyphae seldom grew vigorously over the slide surface. Often these mycelia protruded from humus pellets in the form of dense knots, but they most commonly occurred as small, broken fragments scattered freely over slide surfaces, or protruding from particles of organic debris. Dark mycelial fragments were probably non-viable since they were abundant at all depths, including levels in the A₂ horizon where only three species were consistently and frequently isolated (Sewell, 1959). This apparent difference in distribution from that of hyaline mycelia may result from the thick-walled hyphae being more resistant to decomposition and also more readily observable; whilst the mass of dark mycelial fragments could represent the activity of these fungi over a long period of time, the evanescent nature of most hyaline mycelia was noted on slides which had been immersed for only 7 days.

These dark mycelia, thick-walled, septate and yellowish or reddish brown in colour, were similar to a few of the 'DS' forms isolated on soil plates, but the majority were different from the mycelia of any fungi isolated.

OCURRENCE AND ACTIVITY OF FUNGI IN SOIL

The results of all isolations are summarized in Table 4, where the numbers of species from the major groups of fungi are listed under the methods by which they were isolated. From a consideration of these data and also of the results of direct observations, an attempt is made below to derive an outline of the patterns of growth and activity for some of the fungi in this heathland soil. These suggested growth patterns are based on the methods of study employed, and the degree to which they reflect the true modes of life of fungi in soil is open to question. Nevertheless, this is perhaps as far as any synecological study can, at present, progress. The value of such speculations must rest mainly in providing a tentative foundation on which to base more detailed, autecological studies—which for saprophytic soil fungi are regrettably few.

All seven *Mortierella* spp. were isolated by direct methods; three were also isolated on soil plates, three occurred on root surfaces and none was isolated from dung. Although there was marked seasonal variation in the isolation of *Mortierella* spp. in immersion tubes (Fig. 1), they were observed to grow over Rossi-Cholodny slides immersed during periods when they were not isolated; it is most probable, therefore, that the vigorous colonization of immersion tubes by *Trichoderma* in the warmer months precluded

Table 4. Numbers of species from the dominant groups and genera isolated by various combinations of methods

Method of isolation		D	D	D	D	D	D	D	D	S	S	S	S	S	S	R	R
Direct methods (D)																	
Soil plate (S)						S	S	S	S								
Root plate (R)				R	R	R	R								R	R	R
Dung plate (F)			F	F			F	F				F	F				F
	Total no. species																
Dominant groups of fungi																	
<i>Mortierella</i> spp.	7	3	—	—	1	2	—	—	1	—	—	—	—	—	—	—	—
<i>Mucor ramannianus</i>	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—
Other <i>Mucor</i> spp.	4	—	2	2	—	—	—	—	—	—	—	—	—	—	—	—	—
<i>Thielavia</i> spp.	2	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—
Pyrenomycetes	5	—	3	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Sphaeropsidales	4	1	—	—	—	1	—	—	1	—	—	—	—	—	—	1	—
<i>Penicillium</i> spp.	18	—	—	—	—	—	1	—	—	10	—	—	1	2	3	—	—
Other Moniliaceae	9	—	—	1	—	—	1	—	1	6	—	—	—	—	—	—	—
Dematiaceae	5	—	—	—	—	—	—	—	1	1	—	2	—	—	—	—	—
'DS' sterile mycelia	11	—	—	—	—	—	—	—	—	2	—	—	—	—	—	9	—
Basidiomycetes	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Total no. species	67	6	5	3	1	3	2	1	4	21	—	3	3	13	1	—	—

isolation of the *Mortierella* spp. Although widespread mycelia of *Mortierella* spp. were frequently observed on soil slides, their source was never detected nor was there abundant mycelial development from, or within, any particular area. The form of growth suggested that these mycelia were not solely dependent upon localized and specific nutrient substrates, but rather that the soil solution itself sustained them. Only one species, *M. isabellina*, was frequently isolated by the soil-plate method; a relatively low growth rate in soil (as on agar), accompanied by extensive sporulation, may explain this result. Similarly, a rapid growth, particularly over root surfaces, accompanied by vigorous sporulation, readily fits the results of isolations and observations for *M. parvispora*. It is, however, difficult to postulate, from the available data, soil relationships for the remaining members of the genus. Thus, the hypothesis that *M. zychae* colonizes continuous surfaces, or the soils about them, is more or less restricted to these areas and sporulates but rarely, can only be supported in part by direct observation; the rapid growth of this fungus over slide surfaces was invariably accompanied by extensive sporulation. The possibilities exist, of course, that spores of this fungus were inhibited from germinating, or from further developing, on soil plates by low oxygen tension or unsuitability of the media, or that small, torn mycelial fragments were incapable of regeneration. But perhaps more important is the possibility that its isolation by direct methods was associated with these techniques themselves. Thus, either by reduction of the soil fungistatic factor (Dobbs & Hinson, 1953), or by affecting changes in local soil conditions, or by both, the immersion into soil of solid, inert objects might produce a physical 'rhizosphere' within which certain fungi (normally quiescent or sparsely growing) are stimulated to vigorous growth and, consequently, are isolated so frequently by direct methods as to misrepresent their real occurrence.

Mucor ramannianus, like *Mortierella isabellina*, differs from other members of its genus in being a relatively slow growing and vigorously sporing species, and again these growth characteristics were reflected by the isolation results. Failure of the immersion tube method to isolate this fungus has been related to competition of more rapidly growing species in the surface soil; when 'one-sided' immersion tubes were employed, the fungus was readily isolated at the lowest level (3 in.). The apparent increase in frequency of occurrence of *Mucor ramannianus* with depth (Table 3) was in accord with soil plate results, where the frequencies recorded were 7, 60 and 70 % at 1, 2 and 3 in., respectively (Sewell, 1959). It is possible that neither plating nor direct methods readily revealed its presence in the surface soil, where more rapidly growing fungi were common, and the vertical distribution pattern, based on frequency of occurrence, should be viewed with caution. The results indicate that *M. ramannianus* was widespread in this soil, occurring as a freely growing mycelium both in association with roots and also with humus particles. Its frequency of occurrence in the subsurface soil was perhaps over-emphasized by the soil-plate method, and also possibly underestimated by this method in the surface soil.

All other, and more typical, *Mucor* spp. were isolated from soil only by direct methods. Unlike *Mortierella*s, however, there was no reason to believe that the *Mucor* spp. were stimulated into growth by immersed objects, for mucoraceous hyphae were rarely observed on soil slides. While the absence of *Mucor* spp. from soil plates may have been due to reasons similar to those suggested above for the suppression of *Mortierella zychnae*, it is also possibly a reflexion of limited mycelial growth accompanied by sparse sporulation, as has been previously suggested by Messaien (1957).

Mucor spp. were unusual in being among the few fungi for which localized mycelial activity was demonstrated by the burial of immersion tubes in compact groups; such activity, therefore, was possibly limited to small areas of soil surrounding certain isolated, and perhaps specific, nutrient sources. These *Mucor* spp., for instance, were all isolated from rabbit dung. Similar localized mycelial activity was also demonstrated, by the immersion tube group sampling procedure, for the pyrenomycetous fungi, all of which were isolated frequently from dung.

The greatest differential effect of method was demonstrated in isolations of the *Penicillia* (Fig. 2); they were the commonest fungi on soil plates, in numbers both of colonies and of species, yet only *Penicillium namyslowskii* was isolated (infrequently) by the direct methods. *Penicillia* were common on *Calluna* root surfaces and it is probable that, while many colonies may have originated from conidia, some can be attributed to the presence of active mycelia. Harley & Waid (1955) have shown that occurrence of *Penicillia* on root surfaces still remains appreciable, though greatly reduced, after vigorous washing treatments designed to remove all spores. Observations of *Penicillia* on soil slides were few, but in each instance these fungi were confined to specific substrates (frequently animal remains), from which there was negligible mycelial spread but considerable sporulation thereon. If this mode of life is assumed to reflect the general habit of the genus, the isolation and observation results are in conformity;

spores are produced in great numbers from isolated food bases (including loci in the soil and on root surfaces) and on being dispersed, perhaps mainly in soil water, remain dormant unless influenced by other nutrient sources. The very high frequency of isolation of *Penicillia* on soil plates, and the relative infrequency with which they were directly observed, further suggests that the spores remain viable for long periods.

Trichoderma viride was frequently isolated by all methods and its influence on the isolation of other fungi was considerable. The results indicate that this fungus grew vigorously in the soil, particularly in the surface soil, and on root surfaces. Indeed, from results already reported (Sewell, 1959), it appears possible that *T. viride* grew primarily in association with roots, for there was evidence to suggest that it penetrated the deeper soils of the A2 horizon via root 'pathways'. The conclusion that decreased frequency of isolation with depth may largely have been associated with root density conforms with the growth habit suggested by Chesters (1948). Mycelial activity of *Trichoderma* was markedly retarded during the colder winter months; the frequency of its isolation decreased with temperature to a low level in slide-traps and to zero in immersion tubes (Fig. 1, results for January 1954). The fact that this relatively inactive phase was not demonstrated by soil-plate isolations (Sewell, 1959) indicates the production and survival of numerous conidia, or the maintenance of a widespread yet relatively quiescent mycelium, or both.

The most striking feature of this investigation was the great disparity between the results of direct observation and of isolation of dark mycelia. No dark-coloured, slowly growing sterile mycelia (DS forms) and very few dematiaceous fungi were isolated by the direct methods. On soil plates they were rare and on root plates 'DS' mycelia were isolated occasionally, but only in the absence of more rapidly growing fungi. Yet these mycelia, virtually unrepresented by all isolation methods, were the commonest forms observed on soil slides, where they most frequently occurred in association with humus particles or other organic debris. It cannot, of course, be stated with any certainty that the mycelia observed were similar to any of the 'DS' mycelia isolated. Unless they were incapable of growth on the media used, however, it may reasonably be assumed that the observed, dark-coloured, humus inhabiting fungi were slow-growing mycelia, either sterile or of very limited sporing capacity. They readily fit into the class of 'local colonizers' (Chesters, 1949), and, although active and common members of the soil population (perhaps concerned with the final stages of degradation of organic materials), these fungi were not available for study by the methods of isolation employed.

DISCUSSION

By the use of selective isolation techniques complemented by direct observation it was found possible, albeit tentatively, to obtain information concerning spatial relationships of certain mycelia in the soil. Some fungi were considered to be capable of fairly widespread mycelial growth: in the case of *Mortierella* spp. the most vigorous development appeared to take place in soil spaces, presumably under conditions of high humidity.

There was some circumstantial evidence to suggest that *Mortierella* spp. were affected by a physical 'rhizosphere' produced by the immersion of solid objects. Other fungi, e.g. *Pyrenomyces* and *Mucor* spp., were thought to grow to a limited extent from certain food bases which were not of frequent occurrence in the habitat studied, while *Penicillia* were probably more or less restricted to their food bases. In the latter group negligible mycelial spread was accompanied by vigorous sporulation, but it seemed that sporulation did not necessarily preclude free mycelial development (Garrett, 1956); thus, *Trichoderma viride* appeared to possess both properties. Although mycelial growth of *T. viride* may have been extensive, it is more probable that this fungus grew to a limited extent from food bases (perhaps largely roots—in various stages of development or deterioration) which were very numerous in the surface soil.

The extent to which fungi are capable of spreading from food bases, rate of spread, and also the frequency of such food bases, are probably the major factors determining the range of species isolated by direct methods. Within this range other subsidiary factors affect the species isolated; these include the method itself—or the manner in which the agar medium is presented—the nature of the agar medium, the true depth, time and position of sampling and interactions between these factors.

Generally, the slide-trap appeared to be somewhat more efficient than the immersion tube in isolating species of relatively low growth rates, as was demonstrated by isolations of *Penicillium namyslowskii*, *Mortierella isabellina*, *Phoma* sp. C22, the sterile, basidiomycete mycelium C34, and also *Trichoderma viride* during periods of low temperature.

Growth rate was probably the major factor affecting soil-plate isolations also, but, in this method, growth on the isolating medium (rather than in soil prior to isolation) is the determining factor. Hence the primary selection occurs at a stage later in the isolation process. The results of soil-plate isolations suggested that this relatively retarded selection favours fungi of medium growth rate (e.g. *Penicillia*) which were mainly present in soil as abundant propagules (Warcup, 1951) no less than those of extremely rapid growth rate, e.g. *Trichoderma viride*, *Absidia orchidis*, *Bactridiopsis* sp. C21.

Two broad groups of fungi appeared to be represented in this study: those of very restricted growth in soil but possessing a high sporing capacity, which dominated soil-plate isolations, and those capable of mycelial extension from a food base, e.g. *Mucors*, *Mortierellas* and *Pyrenomyces*, which were commonly isolated by direct methods, but, with two specific exceptions, rarely occurred on soil plates. It may be significant that protoplasmic streaming is limited to the second group; Schütte (1956) has demonstrated a close relationship between this phenomenon and the ability to translocate certain nutrients.

Most fungi which were isolated are cosmopolitan species belonging in the class of 'sugar fungi' (Burgess, 1939). Little chance is afforded by either plating or direct methods for the isolation of fungi of slow growth rate, possessing limited or no sporing capacity; it was demonstrated by direct observation that a section of the fungus flora, largely 'local colonizers' inhabiting humus, was unrepresented in these isolations although they are

perhaps more truly and exclusively soil inhabiting fungi than the majority of those currently included in this class. The failure of the methods used to capture the 'local colonizers' must stand as their major defect. Whenever a number of fungi are brought into contact with an agar medium, a few will develop to the exclusion of the rest. Overwhelming advantage is given to the most rapidly growing and aggressive species: this is irrespective of whether growth originates from reproductive propagules or from established mycelia. By such methods as those outlined by Harley & Waid (1955), Chesters (1948) and, in particular, by Warcup (1955), in which the aim is to isolate the fungi separately *before* they are introduced into the agar medium, our present appreciation of these soil fungi may be greatly extended.

The higher plant community may be defined as a collection of plants growing together and having a 'certain unity' which Tansley (1949) considered to be, in its simplest form, a common habitat—the soil. The determination of a comparable micro-fungal community *growing* and *flourishing* in a common habitat, however, presents considerable technical problems. Fundamentally the difficulty lies in selecting those fungi which are active in the soil rather than those which are merely activated by or during the isolation procedure. The importance of this selection has been greatly emphasized by the demonstration of a widespread soil fungistasis (Dobbs & Hinson, 1953). In past and present synecological studies by 'mass isolation' methods, the dominance of 'sugar fungi' suggests that the common habitat may be provided at least as much by the isolation medium as by the particular soil under investigation. Consequently many differences between soil fungal communities may be masked by the induced, profuse development of ubiquitous species which, in fact, may be relatively rare and ephemeral members of active communities in certain soils.

This investigation was carried out initially during the tenure of a Postgraduate Studentship at Royal Holloway College, University of London, and later on a Nature Conservancy Research Studentship. I wish to express my gratitude to Prof. F. W. Jane, D.Sc., and Dr M. P. Topping for their help and guidance. I am indebted to Mrs M. Turner, Dr A. Apinis, Dr M. B. Ellis, Mr E. W. Mason and Mr G. Smith who kindly identified representative cultures of fungi. The work described forms part of a thesis presented for the Ph.D. degree in the University of London in 1954.

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(Accepted for publication 15 November 1958)

TESTING FOR RESISTANCE TO *POLYSPORA LINI* LAFF. IN FLAX BREEDING

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A pot technique by which the resistance of flax plants to stem-break can be determined within one growing season is described. Since all the inoculated plants develop the seedling phase of the disease, irrespective of whether they later show resistance or susceptibility to stem-break, it is ensured that they are all exposed to similar chances of developing stem-break. The technique is unsuitable for testing resistance to the browning phase, which can be satisfactorily determined in field experiments. The pot technique provides a more exacting test of resistance to stem-break than can be applied under field conditions; varieties showing resistance in the pot test are resistant in the field.

Incidence of stem-break in a susceptible flax variety was unaffected when seedlings were inoculated at different stages of development. Wide variations in the air temperature at which plants were incubated after inoculation also brought about no effects. Development of stem-break was, however, greatly influenced by conditions during growth of the plants, the highest levels of attack being associated with high air humidity.

INTRODUCTION

The disease of flax caused by *Polyspora lini* Laff. exhibits three well-defined phases. The seedling phase appears in the field 3 or 4 weeks after sowing and results in death of the cotyledons. About a month later the stems break just above ground level; this is known as the stem-break phase. Shortly before the plant is mature, the browning stage appears in the form of lesions on sepals, stems and leaves, resulting in the appearance of brown patches in the crop. At this time the disease spreads rapidly and, since at each point where a lesion occurs on the stem the fibre is weakened, the yield of long fibre can be greatly reduced.

It was reported by Lafferty (1921) and Baylis (1941) that when drops of a spore suspension were placed on cotyledons, or when these were smeared with spores, infection resulted and symptoms of stem-break developed later. When the writer repeated these experiments he found that infection was rather erratic, being greatly influenced by environmental conditions. Lafferty (1921) also reported that if mature plants were inoculated with a spore suspension and then incubated in a moist atmosphere, symptoms of the browning phase developed. He further demonstrated that browning appeared when mature plants were inoculated under field conditions. It is, however, difficult to maintain in a moist atmosphere such large numbers of mature plants as are of necessity required in breeding programmes. Moreover, the success of inoculations made in the field depends greatly on weather conditions. Bolsumova (1935) found that

seeds atomized with a spore suspension produced large numbers of infected plants. In the writer's experience, the number of plants developing symptoms of any phase of the disease following upon artificial contamination of the seed is greatly influenced by weather and growing conditions.

In studying the reaction of flax varieties to *P. lini* in the field, Colhoun (1948) sowed seed of the varieties under test in drills alternating with others in which a large proportion of the seed sown was naturally contaminated with the fungus. Both the stem-break and browning phases developed on the plants produced from the contaminated seed, and browning spread readily to the plants of susceptible varieties being tested, so that an estimate of resistance to this phase could be obtained at the end of the first growing season. The reaction of varieties to stem-break could be studied only by saving seed at the end of the first season and sowing it in small plots during the following year. Since the seed harvested was always heavily contaminated, the seedling phase appeared in the second year and stem-break readily developed in susceptible varieties. This, however, meant that the minimum time required to make observations on the reaction of varieties to stem-break was two growing seasons. The method is therefore not entirely satisfactory for testing resistance to stem-break in breeding programmes, on account of the time taken to procure results.

An ideal method of testing material for resistance to *P. lini* in breeding programmes should (a) render it possible to make an assessment within one growing season so that resistant plants may be used in that season for breeding, (b) be applicable to fairly large numbers of plants, (c) reduce to a minimum the number of non-resistant plants escaping infection. Since none of the methods previously employed conformed to these requirements, it was attempted to devise a more satisfactory technique, which could then be applied to material obtained during a breeding programme being carried out in collaboration with the staff of the Plant Breeding Division of the Ministry of Agriculture.

METHODS

In the earliest experiments made in a glasshouse, infection occurred more uniformly if the cotyledons were injured before being inoculated. Under cooler conditions infection occurred readily when drops of a spore suspension were placed on uninjured cotyledons but even here it did not appear as regularly as when the cotyledons were injured. Accordingly, in most of the later experiments, the cotyledons were injured before inoculation by pricking them with a needle in three or four places while they were supported on a glass slide. Using a dropping bottle, a large drop of concentrated spore suspension was then placed on each cotyledon, the suspension being prepared from colonies grown for 10 days on 2 % malt-extract agar. A mixture of spores from colonies derived from as many different sources as possible was always used. All seedlings involved in a single experiment were inoculated on the same day. After inoculation, the seedlings were incubated for 3 days in a moist atmosphere supplied by inverting over each pot a water-tight tin lined with moist blotting-

paper, the tin being of such a diameter as to be supported by the rim of the pot. The seedlings and blotting-paper were sprayed with water daily if necessary. After incubation the tins were removed and the seedlings allowed to dry off slowly. The pots were then moved into their permanent quarters in the glasshouse or in the open air. In the open air the pots were placed in trenches, two rows of pots per trench, with a path 2.5 ft. wide between trenches.

The seedling phase developed on inoculated plants irrespective of whether they later showed resistance or susceptibility to stem-break. When the seedling phase was well developed, an inspection was made to ensure that both cotyledons on each inoculated plant were infected. Only occasional cotyledons which had escaped injury were found to be non-infected, and the plants bearing them were removed. Good conditions for growth being provided, seedlings were not killed as a result of attack in the seedling stage. The stem-break phase had usually developed by the time the plants were flowering and prior to its appearance the total number of plants per pot was recorded. At frequent intervals the number of plants showing stem-break in each pot was recorded, and at maturity the percentage showing stem-break was calculated on the basis of the total number of plants surviving immediately before symptoms of this phase appeared. The browning phase did not develop on any plant during the progress of the experiments.

Unless otherwise stated, the flax variety employed was Stormont Gossamer, which is very susceptible to all phases of attack by *P. lini*. The seed of any commercial variety used was always the product of a single plant culture. Throughout the work 8 in. clay pots were employed, 50 seeds being sown in each pot. Two pots constituted a unit in most experiments.

The air temperatures around pots in glasshouses were measured by self-recording thermometers, the values recorded at intervals of 2 hr. being averaged to give the mean for any period. The mean temperature for open air conditions was obtained by averaging the maximum and minimum temperatures recorded daily. A whirling hygrometer was employed to measure air humidity.

In the early stages of the work, no fertilizers were added to the soil in which the plants were grown. It was, however, soon recognized that the phosphorus content of the soil greatly influenced the susceptibility of plants to stem-break (Colhoun, 1957) and thereafter the following dose of fertilizers was evenly mixed through the soil in each pot: sulphate of ammonia, 3 g.; superphosphate, 8 g.; sulphate of potash, 4 g.; dried blood, 3 g. and calcium carbonate, 6 g. The addition of these fertilizers resulted in good growth and ensured a constant high level of the incidence of stem-break in a susceptible variety. In experiments involving study of the effects of plant nutrition, a compost was prepared by mixing equal volumes of a poor soil, sand and horticultural peat. To this basic compost was added 1 part of calcium carbonate to 500 parts by weight of compost. Other fertilizers were then added as required.

RESULTS

Varietal reaction to stem-break

The reactions to stem-break of thirteen varieties and selections were tested in 1956, the growing period being from 14 May to 27 August. The seedlings were inoculated on 29 May, incubated for 3 days at 13° C. and dried off gradually for 2 days but otherwise they were maintained in the open air. The results obtained (Table 1) can be compared with the reaction to stem-break of each variety on the basis of field observations (Colhoun, 1948).

Table 1. *Varietal reaction to stem-break in pot and field tests*

Variety or selection	Mean % plants developing stem-break in pot test	Reaction in field test
Concurrent (F)*	100	R
La Plata (selection BV) (O)*	100	—
Stormont Gossamer (F)	99	VS
Bison (O)	98	VS
Liral Duke (F)	97	VS
U.S.S.R. No 1 (F)	95	—
Giza Purple (F)	94	S
Textilshchik (F)	93	—
Hindi (O)	80	R
La Plata (selection LB) (O)	71	—
La Plata (O)	46	R
La Plata (selection NB) (O)	34	—

* F = fibre variety; O = oil variety (linseed)

These results demonstrate that the pot test provides a more exacting test of resistance than the field test and that varieties which show resistance (R) in the field do not always exhibit this in the pot test. On the other hand, varieties shown to be susceptible (S) or very susceptible (VS) in the field exhibit a high degree of susceptibility in the pot test.

Confirmation of the above results was obtained when a similar experiment was made in 1957. In that year it was also shown that the following varieties were very susceptible in the pot test: Herkules, J. W. S., Linkopis, Liral Prince, Norfolk Earl, Norfolk Queen and Wiera. It is of interest to note that, of these varieties, Herkules and Linkopis had shown resistance in field tests.

It has already been demonstrated that the susceptibility of the variety Stormont Gossamer is increased by increasing the phosphorus content of the soil whereas no change results from variation in the content of nitrogen or potassium. In two experiments the varieties Concurrent, Noblesse, Norfolk Queen, Textilshchik and Wiera showed the same reaction to phosphorus as Stormont Gossamer. Norfolk Prince was, however, very susceptible to stem-break even in phosphorus-deficient composts, and its susceptibility was unaffected by heavy doses of superphosphate.

Age of seedlings at inoculation

Two pots were sown daily over a 7-day period and the seedlings produced were all inoculated together. The cotyledons of the youngest

seedlings were well developed at the time of inoculation but the foliage leaves showed only a slight protuberance between them. In the oldest seedlings, the shape of the first two foliage leaves was clearly visible and at least three other foliage leaves could be partly seen. After inoculation the plants were incubated in a moist atmosphere at 13° C. for 3 days, for a further day at 13° C. after removal of the pot coverings, then held at approximately 16° C. for a day and finally moved to the open air, where they had been growing up to the time of inoculation. The growing period was from 22 May to 14 August 1956. No differences associated with age of seedlings were observed in the incidence of stem-break, which developed on at least 95 % of the plants produced from each sowing.

Temperature and humidity

The effect of the temperature at which seedlings were incubated after inoculation was studied by growing plants in the open air, and, immediately after inoculation, incubating two pots at each of five different temperatures under damp conditions for 3 days. The plants were then allowed to dry off at *c.* 16° C. for 2 days and returned to the open air. The growing period was from 18 May to 9 August 1956. The results obtained (Table 2) do not indicate that incidence of stem-break was influenced by temperature of incubation.

Table 2. *Incidence of stem-break in relation to temperature of incubation*

Mean temperature during incubation (°C.)	Temperature range (°C.)	Mean % plants developing stem-break
13	12-16	99
17	Constant	95
18	Constant	100
18	10-33	97
22.5	18-35	100

When plants were maintained in a glasshouse throughout the growing period, apart from incubation at 13° C. after inoculation, severe attacks of stem-break did not develop. An experiment was therefore made in which similar sets of plants were grown in the open air and in a glasshouse. All the plants were inoculated on the same occasion and then incubated for the usual period, five different temperature ranges being employed. The cotyledons of half the plants in each set were uninjured at time of inoculation. When the seedlings had become dry after incubation, they were then returned to the conditions under which they had been growing before inoculation. The mean air temperature during the growing period (20 May-24 August 1955) was 15.0° C. (range 4-25° C.) in the open air and 18.2° C. (range 9-38° C.) in the glasshouse (Table 3).

These results demonstrate that when plants were maintained under open air conditions, apart from the period of incubation, the incidence of stem-break was higher than when they were maintained in the glasshouse, where the temperature was higher and the humidity lower than in the open air. The results confirm that the temperature during incubation did

Table 3. *Incidence of stem-break in relation to growing conditions and temperature during incubation*

	Mean and range of air temperature during incubation (°C.)	Mean % plants developing stem-break	
		Cotyledons injured	Cotyledons not injured
Open air	12.2 (9-18)	100	100
Open air	15.5 (14-18)	100	79
Open air	15.7 (12-26)	98	98
Open air	16.4 (14-22)	100	94
Open air	25.3 (12-39)	100	70
Glasshouse	12.2 (9-18)	43	60
Glasshouse	15.5 (14-18)	64	35
Glasshouse	15.7 (12-26)	47	50
Glasshouse	16.4 (14-22)	70	35
Glasshouse	25.3 (12-39)	64	51

not influence incidence of stem-break. Very few plants failed to develop stem-break when maintained in the open air if the cotyledons were injured at inoculation.

A third experiment was made in which plants were grown and incubated under three sets of conditions provided by the open air and two glass-houses. The mean air temperatures for the entire growing period (18 May-9 August 1956) and for the incubation period under each set of conditions are given in Table 4, together with the mean percentage plants developing stem-break.

Table 4. *Incidence of stem-break in relation to growing and incubation conditions*

Mean temperature during growth (°C.)	Mean temperature during incubation (°C.)	Mean % plants developing stem-break
13.1 (open air)	13.1	98
18.0 (glasshouse)	19.5	18
21.7 (glasshouse)	23.7	42

Since variation in the incubation temperature within the range employed can be neglected so far as incidence of stem-break is concerned, it can be concluded from the data that conditions during growth exercise an effect on development of the disease. Fewer plants developed stem-break in the glasshouses than in the open air, where the humidity was higher. It was also observed that the air humidity in the cooler glasshouse, where the lowest number of plants contracted the disease, was lower than in the other glasshouse.

A larger scale experiment was made from 17 June to 20 September 1957 employing sixteen pots under each of three sets of growing conditions: (1) open air with fairly high air humidity and low temperature; (2) glasshouse (a) with a higher temperature and lower air humidity than the open air; and (3) glasshouse (b) with a similar temperature but higher air humidity than in glasshouse (a). The higher air humidity in glasshouse (b) was induced by a spray of water falling continuously on sacking

suspended from the bench and reaching into large trays of water on the floor. The air humidity of this glasshouse was thus increased so that at times it was equal to that of the open air, but frequently it was intermediate between that of glasshouse (a) and the open air. The mean value for the relative humidity in the open air during the period was 85 % (range 50–100 %) when values based on daily determinations at 9.00 a.m. were used. The plants were maintained under one of the above sets of conditions, except during the incubation period after inoculation when they were all held at a temperature of 14° C. Immediately after incubation all the seedlings were allowed to dry off under similar conditions (Table 5). Statistical examination of the data showed that less stem-break developed in glasshouse (a), where the air humidity was lowest, than in either the open air ($P < 0.001$) or in glasshouse (b) ($P < 0.01$). Less stem-break also developed in glasshouse (b) than in the open air ($P < 0.01$). These results indicate that air humidity is of greater importance than temperature in so far as incidence of stem-break is concerned.

Table 5. *Incidence of stem-break in relation to temperature and humidity*

	Mean temperature (°C.)	Mean % plants showing stem-break
Open air	13.8	98.9 ± 0.60
Humid glasshouse (b)	17.2	88.0 ± 3.04
Dry glasshouse (a)	17.8	70.7 ± 3.28

RECOMMENDED TECHNIQUE

Seedlings should be grown in pots of a size suitable for convenient handling and large enough to permit the plants to grow to maturity. Fifty seeds can be safely sown in a pot of 8 in. diameter. The soil should be rich in phosphates and well supplied with all other essentials for good growth.

The seedlings should be inoculated when the cotyledons are well developed and if possible before the shape of any foliage leaves can be clearly recognized. A suitable stage should be reached in from 9 to 16 days after sowing, depending on soil moisture and temperature. The cotyledons should be injured immediately before inoculation, which is satisfactorily carried out by placing a drop of spore suspension on each cotyledon. After inoculation the plants should be incubated for 3 days in a moist atmosphere at a temperature of 13–15° C., then allowed to dry for one day at the temperature of incubation and for a further day at about 16° C. The plants should then be moved to their permanent quarters.

It is desirable that during growth the air humidity should be very high. Under Northern Ireland conditions the plants may remain out-of-doors except during incubation and subsequent drying off, but in drier climates it may be desirable to ensure that the air humidity is increased above that of open air conditions.

Since physiologic specialization may occur in *P. lini*, it is desirable that a mixture of spores obtained from colonies derived from as many different sources as possible should be used for inoculating plants. All the cultures

employed should have been recently isolated from plants or seeds, to ensure that their powers of pathogenicity are as high as possible.

Symptoms of the seedling phase of the disease should appear from about 9 days after inoculation. The cotyledons may be killed about 8 days afterwards and stem-break should make its first appearance about 5 weeks after inoculation.

In applying the technique, it has proved possible for a single worker to inoculate 7500 plants daily when three assistants were available to injure the cotyledons and make all necessary preparations.

DISCUSSION

The above results demonstrate that resistance of flax plants to stem-break can be readily determined by a pot technique which has already been successfully utilized in a programme of flax breeding. This technique exposes all the plants under test to similar chances of developing stem-break, since all of them exhibit the symptoms of the seedling phase. When a susceptible variety such as Stormont Gossamer was inoculated with a spore suspension derived from cultures of different origins, nearly all the plants developed stem-break in all experiments where suitable conditions were provided. It may therefore be concluded that the technique reduces to a very low level the possibility of susceptible plants escaping stem-break. This technique does not enable plants to be tested for resistance to the browning phase of the disease, which can be best determined under field conditions. The non-development of the browning phase in pots even after severe attacks of stem-break can possibly be attributed to the unsuitability of the micro-climate among the plants. Much better ventilation is provided among the plants growing in pots, as compared with that in a crop in the field. The combination of pot and field methods of testing enables the resistance of plants to both the stem-break and browning phases to be determined within one growing season.

The writer wishes to express his thanks to Prof. A. E. Muskett for his interest in the work. Thanks are also due to Mr E. V. B. Wilson for providing seed of many varieties, and to Mr G. I. Janeway for valuable assistance with the routine work.

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(Accepted for publication 1 December 1958)

PATHOGENIC INTERACTIONS BETWEEN *FUSARIUM OXYSPORUM* AND *FUSARIUM SOLANI* ON PEAS

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(With 3 Text-figures)

The previous observation that severity of wilt caused by *Fusarium oxysporum* f. *pisi* on pea variety Onward was decreased by simultaneous inoculation with the pea foot-rot pathogen, *F. solani* f. *pisi*, was confirmed by inoculation tests in a glasshouse; the decrease was statistically significant and was greater when the amount of *F. solani* in the mixed inoculum was increased. Inoculating plants with *F. solani* before *F. oxysporum* gave less wilt than inoculating plants with both fungi simultaneously. The decrease of wilt also occurred in sterilized soil, showing other soil micro-organisms are not involved in the phenomenon.

Field observations on a wilt-susceptible pea variety growing in infested soil showed that although *F. solani* entered pea roots earlier than did *F. oxysporum*, the plants were killed by the wilt pathogen. Wilt spread less rapidly along rows of peas from foci of inocula containing both *F. solani* and *F. oxysporum* than from foci of *F. oxysporum* alone. Foot-rot also spread less from a mixed inoculum than from *F. solani* alone.

Differences in appearance of the macrospores of the two fungi were considered to be the only reliable taxonomic criteria by which to distinguish between the two species.

INTRODUCTION

Fawcett (1931) emphasized the need for studying multiple infection of plants, and several diseases resulting from two or more pathogens have been described. They may result from simultaneous infection by different pathogens or from successive infections occurring soon after one another. Two pathogens entering a host may act synergistically or one may lessen the damage caused by the other.

Pea wilt, caused by *Fusarium oxysporum* f. *pisi* (Linf.) Snyder & Hansen, is often associated in the field with foot-rot, caused by *F. solani* f. *pisi* (Jones) Snyder & Hansen. During tests of the pathogenicity of many isolates of both fungi, the severity of wilt was decreased when *F. solani* was introduced into the soil at the same time as *F. oxysporum* (Buxton, 1955). This paper describes experiments made in the field and in the glasshouse to determine the extent of the interaction between the *Fusaria* on peas, and an accompanying paper (Perry, 1959) deals with the possible mechanisms underlying it at each different stage of the infection process.

TAXONOMIC AND MORPHOLOGICAL DISTINCTIONS BETWEEN *FUSARIUM OXYSPORUM* F. *PISI* AND *FUSARIUM SOLANI* F. *PISI*

Because of difficulties in their taxonomic separation, the fungi that cause wilt and foot-rot of peas have often been confused, and it was considered

necessary to investigate their differences. Members of the genus *Fusarium* have created many taxonomic problems, and at present there is little or no agreement between various authorities. Not only do difficulties arise from the scarcity of definite cultural characters that could be used to separate the different species, but the genetic mechanisms possessed by any single species (Buxton, 1956) can lead to considerable variability.

Wollenweber & Reinking (1935) divided the genus into 16 sections, with 65 species, but Snyder & Hansen (1940, 1941, 1945) condensed it into 9 species, with no sections. Gordon (1952) re-classified parts of the genus and re-erected 9 sections, but Carrera (1954) in Argentina, and Bilai (1955) in Russia, have since re-adopted most of the Wollenweber system. Wollenweber & Reinking's sections *Elegans* and *Martiella* contain *F. oxysporum* and *F. solani* respectively, and the essential differences between these two species, according to them, can be summarized as follows:

Colour of stroma	Sclerotia	Pionnotes (masses of spores in slime on surface of culture)	Macrospore shape
<i>F. oxysporum</i> Brownish white to violet	Blue or green	Formed later, not common	Weakly pedicellate, generally 3-septate, curved or almost straight, septa weakly defined
<i>F. solani</i> Brownish white, yellow, green or dark brown	Blue or green	Common	Weakly pedicellate, generally 3-septate, spindle-shaped, slightly curved, septa thick, well-defined

Characters other than the shape of spores and conidiophores have been found unreliable by Snyder & Hansen (1954), Buxton (1955) and Reid (1958). Sherbakoff (1953) found difficulties in discriminating between *F. oxysporum* and *F. solani* isolated from the surface of citrus roots, and Miller (1946) considered that the pea root-rot *Fusaria* were morphologically so similar to the *Elegans* *Fusaria* that he included them in that section. Our conclusion, gained from examining many isolates of *Fusaria* from diseased peas in Britain, is that the shape of the macroconidia is the only reliable morphologic difference between the *Fusarium* causing wilt and that causing foot-rot (Fig. 1).

The isolates of *F. solani* f. *pisi* consistently differ from those of *F. oxysporum* f. *pisi* in having macrospores with (1) a broader distal cell, giving a blunter appearance, (2) thicker and more highly refractive septa. The cytoplasm of end cells of the macroconidia was as granular in *F. solani* as in *F. oxysporum*, an observation which differs from Sherbakoff's description of his isolates. But in agreement with earlier descriptions of *F. solani*, our isolates produced macroconidia, mostly in pionnotes, more frequently than did *F. oxysporum*. When cultures from diseased plants were made on potato-dextrose agar, their colour varied. Isolates of *F. oxysporum* were white, red, vinaceous, purple or blue-black and those of *F. solani* were white, yellow, pink or brick-red. *F. oxysporum* frequently developed a dense cottony aerial mycelium, whereas *F. solani* grew less rapidly and produced a more diffuse and sparse aerial mycelium, often with pink or blue-green pionnotes.

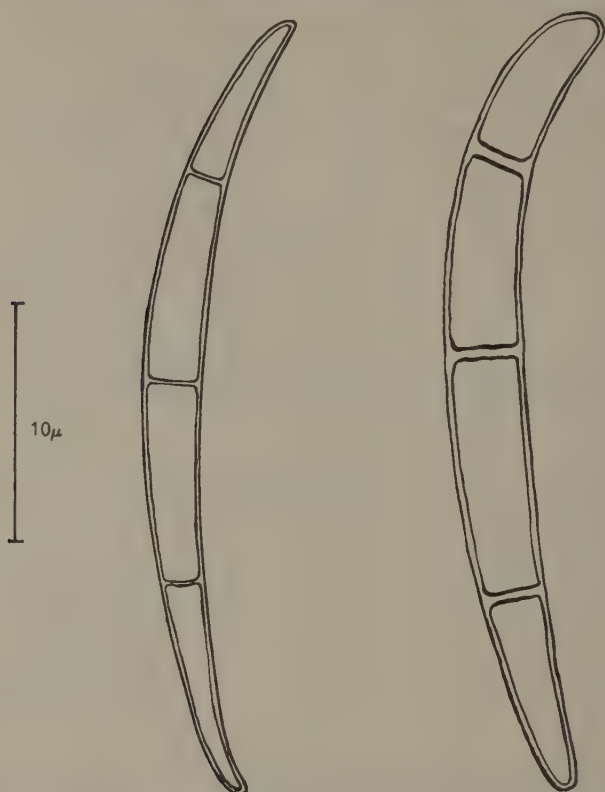


Fig 1. Macrospores of *F. oxysporum* (left) and *F. solani* (right) isolated from diseased peas.

DEVELOPMENT OF *FUSARIUM OXYSPORUM* AND *F. SOLANI* IN DISEASED PLANTS IN THE FIELD

In 1957 and 1958, samples were taken from a crop of peas, variety Onward, growing in Essex on wilt-infested land. All the plants in each sample were measured and the degree of wilting and severity of foot-rot was noted. Fragments 5 cm. long, measuring from the hypocotyl, were taken either from the stem or the root, and the cortex was separated from the stele. Both the stele and a strip of cortex were cut into 1 cm. sections, surface sterilized in 1 % silver nitrate for 1 min., immersed in 20 % sodium chloride and then washed in three changes of sterile distilled water before plating on potato-dextrose agar. After 5 days' incubation at 25° C., the plates were held in daylight at room temperature for 2 days, after which the fungi present were identified. In 1957 only the stem was plated out, but, in the first three samples of 1958, only the root was plated, to discover when invasion occurred and the extent to which the root was invaded before the stem was colonized. In later samples, however, only the stem was plated out, as in the first year. Peas were sown on 25 March, and in samples 4-6 the peas were at early-flowering, late-flowering and pod-setting stage respectively (Table 1).

Table 1. *Development of wilt and foot-rot in pea variety Onward at Rayne, Essex, in 1958*

	Samples					
	1	2	3	4	5	6
Days after sowing	35	50	64	77	92	107
Average height of plants (cm.)	10	15	30	45	54	58
Degree of wilting (Complete wilt = 4.0)	—	—	0.1	1.4	2.6	3.1
Severity of foot-rot	—	—	—	Slight	Moderate	Severe
	Root sections			Stem sections		
Percentage plants infected by <i>F. oxysporum</i>	—	6	31	70	100	97
Percentage plants infected by <i>F. solani</i>	—	56	75	45	47	64

Note. The degree of wilting was estimated on a 0-4 basis, 4 representing all leaves on a plant wilted; *F. oxysporum* and *F. solani* were identified using the criteria given above.

The results obtained in 1957 were similar to those given in Table 1 for 1958, except that foot-rot symptoms were less severe in 1957, which was drier. In 1958, wilt symptoms developed rapidly when the plants reached the early-flowering stage, and at this time the numbers of pieces of stem (vascular tract) infected with *F. oxysporum* also increased. Thirty-six per cent of the plants in the final sample were dead, having dried out and turned brown. In sample 2, *F. oxysporum* was isolated from roots before wilt symptoms developed, and later, when the plants were beginning to wilt, it was isolated from stems, where it was restricted to the stele, except for a few plants in which it had invaded the cortex. An orange-red discoloration of the stele is usually considered characteristic of infection by *F. oxysporum* (Linford, 1928), but it is not a reliable diagnostic feature; all plants in our samples with discoloured vascular systems were infected, but not all infected plants were discoloured.

Foot-rot symptoms also became more severe as the plants aged, but *F. solani* could be isolated from the cortex of young plants before symptoms appeared. It invaded roots earlier than did *F. oxysporum*, and more pieces were infected by it. In addition, *F. solani* was often isolated from the root stele as well as from the cortex, but rarely from the stem vascular tissue. At the later sampling times, although 89 % (sample 5) of the plants had foot-rot, *F. solani* grew from only 47 % of the total, and *Ascochyta pinodella*, which also causes a foot-rot of peas, was often isolated alone or together with *F. solani*. One or other fungus developed from 64 % of the total, making a considerable discrepancy between the number of plants with foot-rot symptoms and the number of plants from which foot-rot fungi developed. This may mean either that the fungus responsible was not recovered from all infected plants, or that old plants in the field can have foot-rot from other causes.

It is interesting to note that in both years the morphological features of most of the isolates of *F. oxysporum* from this locality resembled those of

F. oxysporum var. *redolens* as described by Gordon (1952). Seventeen out of nineteen cultures of this fungus were highly pathogenic when inoculated to pea variety Onward.

RATE OF SPREAD OF THE DISEASES FROM FOCI OF INOCULUM IN THE FIELD

To measure the rate of spread of wilt in pea variety Onward, and what effect *F. solani* had on it, inocula of the two fungi were placed at intervals on a plot of ground at Rothamsted, on which peas had never been grown. The inoculum was composed of 1 bushel of wheat grain, 2 bushels of barley grain and 2 bushels of 'Vermiculite', which were mixed together and soaked overnight in cold water; excess water was then squeezed out and the mixture was placed in flat wooden seedling boxes, lined and covered with 'Kraft' paper, before autoclaving, on two successive days, for $1\frac{1}{2}$ hr. at 20 lb. pressure. Later each box was inoculated with 40 ml. dense spore suspension of *F. oxysporum* or *F. solani*. After 3 weeks' incubation at room temperature, two handfuls of inoculum were placed in the middle of 8 ft. long rows in nine plots, each plot consisting of six rows; peas were sown immediately afterwards (22 March). The inocula were *F. oxysporum* alone, *F. solani* alone, both together, and uninoculated mixture as a control. To assess spread of *F. oxysporum* along the rows, the percentage of yellowed and rolled leaves on the plants was recorded each week every $2\frac{1}{2}$ in. (the distance between plants) along each row. For *F. solani*, yellowed leaves were recorded in conjunction with the occurrence of foot-rot lesions (Fig. 2).

Foot-rot began to spread earlier than wilt. By 12 July, wilt had spread further from the inoculum of *F. oxysporum* alone than from the double inoculum; also, *F. oxysporum* had retarded the spread of foot-rot. In similar plots, in which only the middle row was inoculated, there was no spread between adjacent rows, implying that root contact may be necessary for the fungi to move from plant to plant.

INOCULATION EXPERIMENTS IN THE GLASSHOUSE, USING MIXED CULTURES

Similar experiments on the effects of double inoculation were done in an unheated glasshouse, mainly during the summer months. The fungi originating from single spores, were grown in a liquid Czapek-Dox medium at 25 ° C. for 10 days and, before using as inocula, dispersed in a blender (M.S.E. 'Atomix') for 1 min. at high speed.

Peas, variety Onward, were grown from seed in boxes containing a mixture of 4 parts unsterilized loam, 1 sand and 1 peat. When the plants were 2 in. high, they were transplanted into 4 in. pots and inoculated by pouring 10 ml. fungus suspension over the roots before the soil was pressed round them. When using this method, it is essential to determine whether the culture fluid of *F. oxysporum* has any effect on development of wilt. To do this, the fungus inoculum was divided into two parts. One half of it was centrifuged at 3000 r.p.m. for 15 min., the supernatant fluid decanted and the fungus re-suspended in an equivalent volume of distilled water, while the other half was used without removing the culture fluid. Wilt

symptoms developed only 1–2 days earlier in plants inoculated with the fungus in the culture fluid than in plants inoculated with the fungus in distilled water, and similar symptoms were caused by both types of inoculum. This being so, it was not considered necessary to remove the culture fluid from the inocula used in the pot experiments.

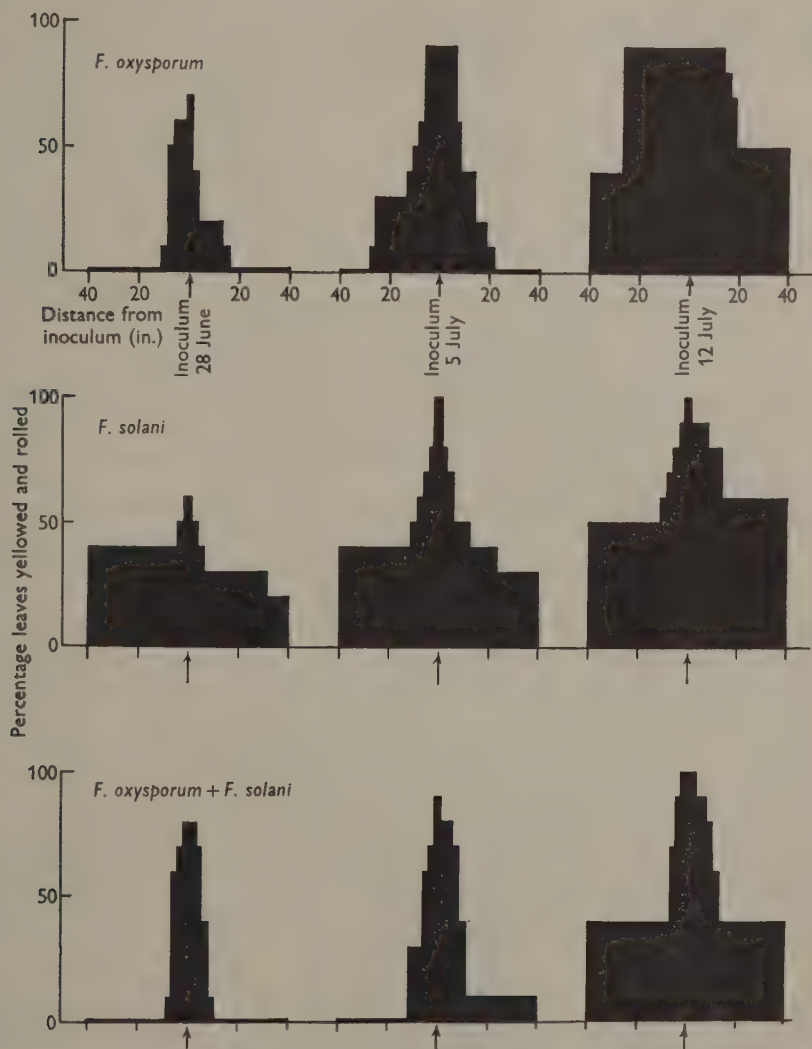


Fig. 2. Rates of spread of wilt and foot-rot, from inoculum, in the field.

The seedlings were inoculated with *F. oxysporum* alone, with *F. solani* alone, or with a mixture containing equal volumes of each. Both inocula containing one fungus alone were diluted with an equal volume of water, to compensate for the dilution of one fungus by the other when both were added together in the double inoculum (Table 2).

Table 2. *Decrease of wilt by co-inoculation with Fusarium solani*

Inoculum	Days after inoculation											
	13		15		17		19		21		23	25
	I	% W	I	% W	I	% W	I	% W	I	% W	% W	% W
<i>F. oxysporum</i>	4/30	3	15/30	7	25/30	25	30/30	31	30/30	57	75	82
<i>F. oxysporum</i> + <i>F. solani</i>	0/30	0	6/30	4	12/30	9	19/30	16	30/30	45	55	66
<i>F. solani</i>	—	0	—	0	—	4	—	6	—	8	16	27
None	—	0	—	0	—	4	—	5	—	6	11	26

I = incidence of wilt symptoms. % W = percentage wilted leaves in each treatment (30 plants per treatment).

Wilt symptoms appeared on the plants inoculated with *F. oxysporum* alone 13 days after inoculation, and appeared 2 days later in those inoculated with both fungi. *F. solani* did not prevent *F. oxysporum* from killing the plants, though death was delayed. Percentage wilt was consistently less in those plants inoculated with both fungi than in those inoculated with *F. oxysporum* alone, but, by the end of the experiment, wilt in both treatments had reached nearly 100 %. Both the controls and the plants inoculated with *F. solani* alone had slight symptoms that resembled wilt, but their vascular tracts were not discoloured, and *F. oxysporum* could not be isolated from them.

When the results are expressed as number of plants with wilt symptoms, it is clear that the onset of wilt was delayed by co-inoculation with *F. solani*, and this was particularly obvious 15 and 17 days after inoculation. This delay was analysed statistically by obtaining a χ^2 value from a 2×2 contingency table, using Yates's correction factor for continuity because of the low class frequencies. Fifteen days after inoculation $\chi^2 = 4.68$ and $P = 0.05-0.02$; 17 days after inoculation, $\chi^2 = 9.95$ and $P = 0.01-0.001$, showing the delay of wilt symptoms to be highly significant.

At the end of the experiment, 5 cm. lengths of root and of stem, measuring from the hypocotyl, were cut from five plants in each treatment; the cortex was separated from the stele, and both were cut into 1 cm. lengths, which were then plated on potato-dextrose agar. In the plants inoculated with the fungi separately, *F. oxysporum* had colonised the stele of stems more extensively than that of roots, and only a few fragments of the cortex of roots were infected. *F. solani* grew mainly from the cortex of roots and stems, though some pieces of the stele from a few plants were infected. Both fungi grew from the plants receiving the combined inoculum. *F. oxysporum* was dominant in the stele of the root and stem, though it occasionally invaded the cortex of severely wilted plants. *F. solani* grew only from the cortex of roots and stem and only from a few of the sections that had foot-rot lesions. Plants taken from the field gave a similar result.

Although in these experiments the severity of pea wilt was only moderately decreased by co-inoculating plants with *F. solani*, the effect is genuine, as similar results were obtained many times. Wilt symptoms always appeared later in doubly inoculated plants than in those inoculated with

F. oxysporum alone. The extent of the decrease varied from one experiment to another, and probably depends on temperature, water supply and host nutrition.

EFFECT OF *FUSARIUM SOLANI* ON PEA WILT IN STERILIZED SOIL

Peas, variety Onward, were grown in compost that had been sterilized at 20 lb. pressure for 20 min. on two successive days. They were inoculated by the method described for the previous experiment and transplanted into sterilized compost (Table 3).

Table 3 shows clearly that *F. solani* delayed wilting by *F. oxysporum* in sterilized soil, just as it had done in unsterilized soil, so that the phenomenon does not depend on the presence of other micro-organisms that normally exist in field soils or in potting compost.

Table 3. *Percentage of wilted leaves 11 and 14 days after inoculation with Fusarium oxysporum and F. solani in sterile soil*

Inoculum	Days after inoculation	
	11	14
<i>F. oxysporum</i>	13	30
<i>F. oxysporum</i> + <i>F. solani</i>	5	20
<i>F. solani</i>	1	12
None	1	13

EFFECT ON PEA WILT OF VARYING THE AMOUNT OF INOCULUM OF *FUSARIUM SOLANI*

F. oxysporum and *F. solani* were cultured in liquid Czapek-Dox medium for 10 days at 25° C., and after dispersion in the 'Atomix' they were centrifuged at 3000 r.p.m. for 15 min. *F. oxysporum* was then re-suspended in a volume of distilled water equal to the original culture fluid and *F. solani* was resuspended in $\frac{1}{4}$ th of the volume of culture fluid. From the concentrate, a dilution series of *F. solani* was made to give 8, 4, 2, 1 and 0.25 times the original concentration of fungus in the culture medium. An equal volume of each dilution was added to a constant volume of *F. oxysporum* to give different ratios of the two fungi, which were then inoculated on to peas, variety Onward. Fig. 3 shows that the extent to which wilt was decreased was correlated with increasing concentrations of *F. solani*.

EFFECT ON WILT DEVELOPMENT OF INOCULATING WITH *FUSARIUM SOLANI* BEFORE INOCULATING WITH *F. OXYSPORUM*

Pea seedlings, variety Onward, were divided into three groups, each containing sixty plants. In the first group, twenty plants were inoculated with *F. solani* when they were 6 days old, and the remaining forty were transplanted without being inoculated. When the plants were 10 days old, those that had been inoculated with *F. solani*, and twenty of the uninoculated ones, were removed from the pots and inoculated with a 10-day-old

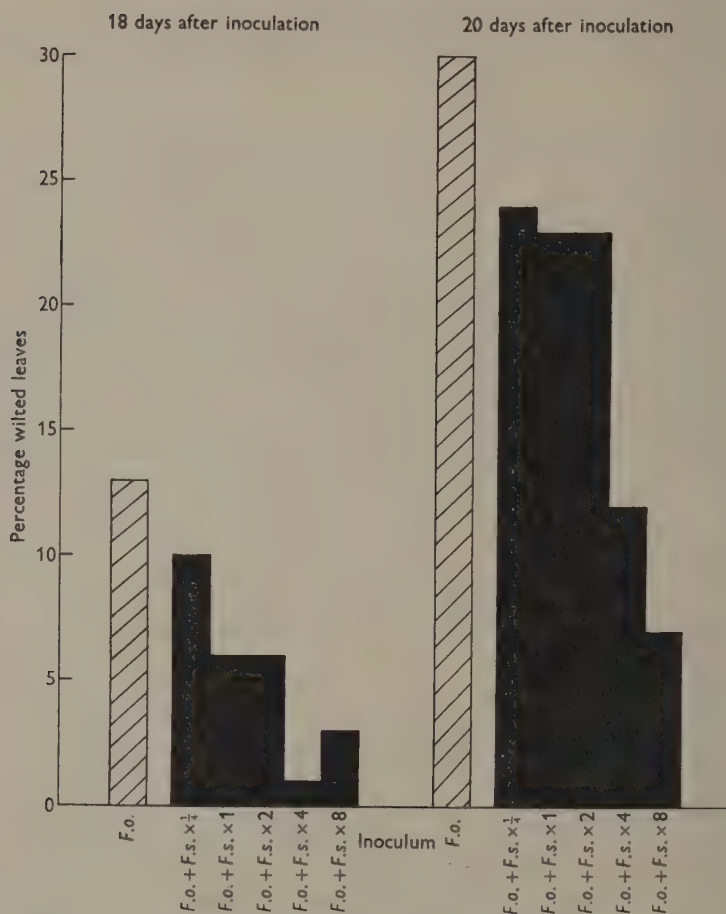


Fig. 3. Effect on pea wilt of varying the amounts of inoculum of *F. solani*.

culture of *F. oxysporum*. They were then replanted in the same soil from which they had been taken. The remaining twenty uninoculated plants were removed from the pots and replanted without being inoculated. In the second group, exactly the same procedure was followed, except that *F. solani* was inoculated on to the plants when they were 8 days old. In the third group, 10-day-old seedlings were inoculated with *F. oxysporum* and *F. solani* simultaneously and with *F. oxysporum* alone. All the plants were therefore at the same age when inoculated with *F. oxysporum* and, within any one set, all the plants were transplanted at the same time (Table 4).

Wilt was less severe in plants inoculated with *F. solani* either 2 or 4 days before *F. oxysporum* (groups I and II) than when both fungi were used together (group III). When *F. solani* is already established in the root tissues it probably alters cells so that the entry of *F. oxysporum* is made more difficult, but it may prevent the development of *F. oxysporum* in the plant by releasing toxins produced only when it grows in pea plants.

Table 4. Percentage wilt 17 days after inoculation in plants inoculated with *Fusarium solani* before, or simultaneously with *F. oxysporum*

	Inoculated with <i>F. oxysporum</i> alone	Inoculated with <i>F. oxysporum</i> (F.O.) and <i>F. solani</i> (F.S.)		Difference between A and B
	A		B	
Group I	43	F.S. 4 days before F.O.	25	18
Group II	40	F.S. 2 days before F.O.	22	18
Group III	47	F.S. at same time as F.O.	35	12

Although our results show that development and rate of spread of pea wilt is slowed when *F. solani* f. *pisi* is present, the effect is hardly large enough to have any practical importance.

This work was done while D. A. Perry was in receipt of a training grant from the Agricultural Research Council.

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(Accepted for publication 21 November 1958)

STUDIES ON THE MECHANISM UNDERLYING THE REDUCTION OF PEA WILT BY *FUSARIUM* *SOLANI* F. *PISI*

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(With 1 Text-figure)

The mechanism underlying the decrease caused by *Fusarium solani* f. *pisi* in the severity of pea wilt due to *F. oxysporum* was studied by examining sites of possible interaction in soil and in host. *F. solani* produced no substance *in vitro* that inhibited spore germination or growth of *F. oxysporum*, and there was no evidence that the two fungi competed to the detriment of *F. oxysporum* either in the rhizosphere of peas or in the soil. Some water extracts of foot-rot lesions contained a substance inhibitory to the germination of *F. oxysporum* spores, but it could not always be demonstrated. No inhibition was caused by exudates or extracts of pea roots infected with *F. solani*.

F. solani colonized the epidermis and outer cortex of pea roots more rapidly and more extensively than did *F. oxysporum*. Three days after inoculation with *F. solani*, pea roots respired 25 % more than uninoculated ones. The physical nature and metabolic activity of the cortex was altered through infection by *F. solani*, so it is concluded that it was these changes alone that delayed the progress of *F. oxysporum* to the stele, resulting in a decrease in wilt.

INTRODUCTION

When *Fusarium oxysporum* f. *pisi* (Linf.) Snyder & Hansen and *F. solani* f. *pisi* (Jones) Snyder & Hansen are inoculated together on to peas, the severity of wilt is significantly less than when plants are inoculated with the wilt fungus alone (Buxton, 1955; Buxton & Perry, 1959). Buxton (1957) showed that there was no interaction between the two fungi on solid agar media and that a culture filtrate of *F. solani* did not inhibit the growth of *F. oxysporum*.

Similar interactions between two *Fusaria* have been described by McClure (1951), working with *F. oxysporum* f. *batatas* and *F. solani* f. *batatas* on sweet potato, by Borlaug (1945) with different biotypes of *F. lini* on flax, and by Graham (1955) between *F. oxysporum* var. *redolens* and *F. moniliforme* on asparagus. None of these workers attempted to elucidate the mechanisms by which the disease caused by one fungus was lessened by the other, although McClure suggested that *F. solani* induced the host to form tyloses which may then have prevented penetration by *F. oxysporum*, and Graham considered that his *Fusaria* competed for available nutrients on or near the root surface.

The experiments described in this paper were designed to discover where

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and how *F. solani* delayed the expression of wilt symptoms. The following possibilities were examined: (1) whether *F. solani* delays invasion of the stele by *F. oxysporum*; (2) production *in vitro* by *F. solani* of a growth inhibitor of *F. oxysporum*; (3) antagonism between the two fungi in the soil and in the rhizosphere of the host; (4) production of substances toxic to *F. oxysporum* by a plant infected with *F. solani*; (5) some change in the anatomy or in the respiration intensity of infected roots.

EFFECT OF *FUSARIUM SOLANI* ON COLONIZATION OF VASCULAR TISSUE OF PEAS BY *F. OXYSPORUM*

Peas were grown and inoculated as described by Buxton & Perry (1959). Twenty plants were inoculated with *F. oxysporum* alone and twenty with *F. oxysporum* and *F. solani* together. Fifteen days after inoculation, when 18 % of the leaves on plants inoculated with *F. oxysporum* alone and 5 % on those inoculated with both fungi were wilted, the plants were removed from the pots and, after washing in running tap water, 5 cm. lengths of root and stem, measuring from the hypocotyl, were cut from them. The stele was dissected from the cortex, cut into 1 cm. sections, and plated out. *F. oxysporum* grew from 40 out of 200 sections from plants inoculated with *F. oxysporum* alone and from 9 out of 200 from plants inoculated with both fungi, i.e. from 20 % and from 4.5 %, respectively. The sections of the stele were plated out in the order that they were taken from the plant. In some roots only one section, often from different positions in different roots, was infected, indicating that *F. oxysporum* can invade the stele at any point along the root. The decrease in number of wilted leaves on plants that received the combined inoculum was therefore correlated with fewer sections infected with *F. oxysporum*, showing that *F. solani* delays invasion of the plant by the wilt fungus.

INHIBITION OF *FUSARIUM OXYSPORUM* BY *F. SOLANI* *IN VITRO*

The possibility that *F. solani* may produce substances *in vitro* that are inhibitory to *F. oxysporum* was tested both by growing the two fungi together on a number of different agar media, and by testing the effects of culture filtrates of *F. solani* on germination of spores of *F. oxysporum*.

The agar media were potato-dextrose, pea extract, and soil extract + root exudate. This last medium was used because it resembled the nutrients available at the surface of pea roots. *F. oxysporum* and *F. solani* were inoculated on to each of the three media on opposite sides of Petri dishes. There was no inhibition of either fungus by the other on any of the dishes; the colonies grew together and their hyphae intermingled freely.

Culture filtrates of *F. solani* were obtained by growing it in pea extract, bean extract, potato extract and malt extract liquid media. After 10 days' incubation, the culture fluid was passed through a Seitz filter. No zones in which spore germination was inhibited occurred when sterile filtrates were tested by putting them into porcelain cylinders on the surface of $\frac{1}{4}$ strength Czapek-Dox agar containing spores of *F. oxysporum*.

INTERACTIONS BETWEEN *FUSARIUM SOLANI* AND *F. OXYSPORUM*
IN THE RHIZOSPHERE OF PEAS AND IN THE SOIL

Although no antagonism between the two fungi could be demonstrated *in vitro*, it might nevertheless occur in soil. For example, Broadfoot (1933) found that among twenty-one cultures of different fungi and bacteria that decreased the virulence of *Ophiobolus graminis* to cereals in soil, six were not antagonistic towards it on potato-dextrose agar. *F. solani* might produce an inhibitor to *F. oxysporum* in soil and not in culture, or it may be a more vigorous soil saprophyte and so exclude *F. oxysporum* by competition for organic substrates in the soil. Furthermore, the conditions in the rhizosphere soil around the roots of peas are not the same as those in the surrounding soil, so that the two fungi might interact differently there.

To test these possibilities, the two fungi were added alone, and together in various proportions, to unsterilized soil in jam jars. Four-day-old pea seedlings, variety Onward, were then planted in some of the jars. Soil samples, taken 0, 2, 4 and 8 days after adding the fungi, were plated-out by the soil dilution plate method on rose bengal-streptomycin agar (Martin, 1950), and the colonies of the two fungi that developed were counted. After 2 days, the pea seedlings were removed from the jars and rhizosphere soil was plated-out.

In all the soil samples, the ratio of numbers of colonies, *F. oxysporum*/*F. solani*, on the dilution plates was similar to that in which they were added to the soil. In addition, the percentage of *F. oxysporum* colonies in the total of both *Fusaria* that developed from the rhizosphere was similar to that from soil which had not been planted with pea seedlings. It seems, therefore, that there was no competition between the two fungi in the soil or in the rhizosphere.

TOXICITY TOWARDS *FUSARIUM OXYSPORUM* AS A RESULT OF THE
INTERACTION BETWEEN *F. SOLANI* AND THE HOST

Because *F. oxysporum* was not affected by *F. solani* in the soil or in the rhizosphere, it seemed that the interaction between them might occur during penetration of the host, or during growth within it. Substrates from hosts infected with *F. solani* might affect growth of the wilt fungus. To test this possibility, both extracts from root tissue and exudates from living roots of plants with foot-rot were prepared.

Root exudates were obtained by growing plants infected with *F. solani* in the apparatus used by Buxton (1957), and were sterilized by passing through a sintered-glass, bacteria-proof filter. Extracts were made from young pea roots 3 days after inoculation with *F. solani*, and from foot-rot lesions on pea stems 6 weeks after, by grinding 1 g. tissue in 10 ml. distilled water. The macerated tissue was then centrifuged and the supernatant fluid sterilized by passage through a sintered-glass filter.

The exudates and extracts were tested for their effects on *F. oxysporum* by the following methods:

(1) They were incorporated into a modified Czapek-Dox agar and the radial growth rate of *F. oxysporum* was measured. The agar contained

only 0.5% sucrose, as a higher concentration might have masked the action of any inhibitor present.

(2) They were added to a modified liquid Czapek-Dox medium, which was then inoculated with *F. oxysporum*. After 10 days' incubation, the mycelium was filtered, washed, dried and weighed, to determine if there was any difference in the mycelial growth in the presence of the exudates or extracts.

(3) Their effects on germination of spores of *F. oxysporum* were tested by putting them into sterile porcelain cylinders on the surface of modified Czapek-Dox agar containing spores of *F. oxysporum*.

(4) They were added to a sterile water suspension of spores of *F. oxysporum* in clean test tubes, and, after 24 hr. incubation at 25° C., the percentage of spores that had germinated was determined.

All the tests on exudates and extracts from young roots showed that there were no inhibitors in plants infected by *F. solani*. Similarly, there was no effect with extracts from foot-rot lesions in the first three tests listed above, although there was some indication, in the fourth test, of an inhibitor of spore germination in water suspensions.

EFFECT OF STERILE WATER EXTRACTS OF STEMS WITH FOOT-ROT LESIONS ON SPORE GERMINATION OF *FUSARIUM OXYSPORUM*

The eight experiments done to determine whether an extract of stems with foot-rot lesions depressed the germination of spores of *F. oxysporum* gave inconsistent results; in three of the experiments spore germination in highly dilute extracts of the stem lesion was less either than that in healthy stem extract at the same dilution or that in sterile water. In two of the experiments, spore germination in stem-lesion extract was less than that in healthy stem extract, but not less than that in sterile water, and in the remaining three, spore germination was the same in both extracts. Fig. 1 shows the results of one experiment that demonstrated the presence of an inhibitor; each of a series of dilutions of stem extract (S/10, S/1000, S/10,000 when S/10 = 1 g. stem tissue in 10 ml. water) was put into four test tubes and spore germination was counted in two samples from each tube.

At S/10,000, germination in extract from stem lesions was significantly ($P < 0.001$) lower than in sterile water or in extract from healthy stem tissue.

These results suggest that there were two factors in extracts from stem lesions, one stimulating spore germination, the other inhibiting it; extract of healthy stems contained only the stimulatory factor, whereas that from diseased stems contained both. At S/10,000 dilution, the stimulatory factor in extracts from both healthy and diseased stems became ineffective, and only at that dilution did the inhibitory factor in diseased stem extract become obvious. The inconsistent results in different experiments become explicable on this hypothesis by postulating that extracts from different diseased stems contain the two factors in different proportions, so that only sometimes is the inhibitor concentrated enough to withstand the dilution necessary to get rid of the stimulant.

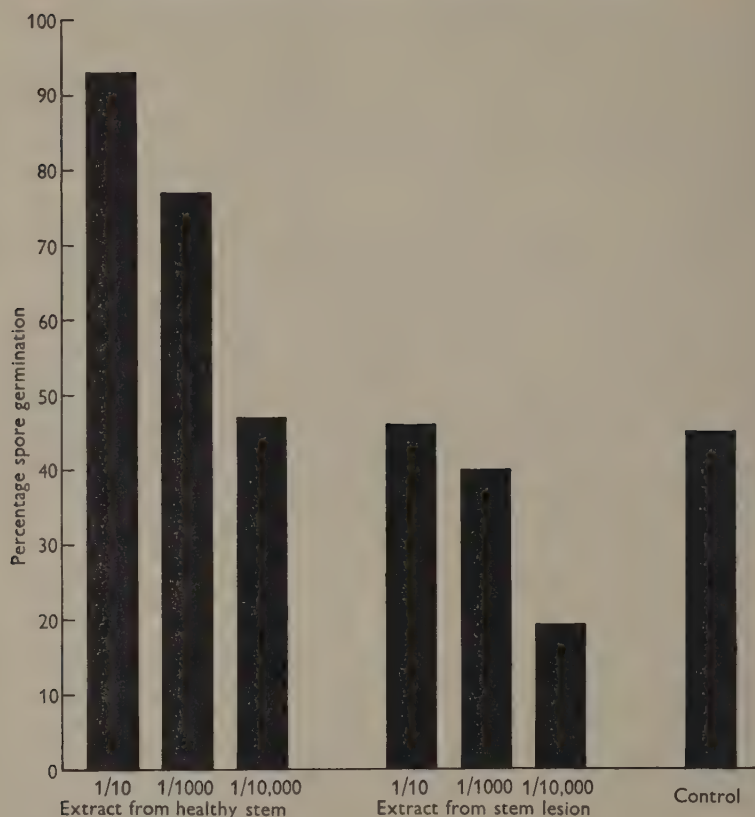


Fig. 1. Effect of sterile water extracts of stems with foot-rot lesions on germination of *Fusarium oxysporum* spores.

INVESTIGATIONS OF THE ANATOMICAL CHANGES OCCURRING IN THE HOST DURING INFECTION

The results from all the previous experiments combine to show that the growth of *F. oxysporum* outside the pea root is not affected by *F. solani* and that, although pea plants infected with *F. solani* may produce substances inhibitory towards *F. oxysporum*, they are not easily detectable. Another approach was therefore made to the problem, to see if there were any morphological changes following infection by *F. solani* and whether these might delay the progress of *F. oxysporum*.

Pea seeds, variety Onward, were germinated on wet filter paper and then placed over damp cotton wool in glass beakers. After 7 days they were planted in moist unsterile loam in jam jars and inoculated either with *F. oxysporum* or with *F. solani*, by pouring 5 ml. distilled water suspension of fungal mycelium and spores over the roots. The suspension was obtained by culturing the fungi in liquid Czapek-Dox medium for 10 days at 25° C., centrifuging for 15 min. at 3000 r.p.m. and re-suspending in an equivalent volume of distilled water. Peas were sectioned 1, 2, 4 and

6 days after inoculation, after they had been washed carefully in running tap water to remove the soil. The roots were supported by 1 % sodium alginate, sectioned at 15–20 μ with a freezing microtome, and mounted in 0.5 % cotton blue in lactophenol.

Hyphae of *F. solani* were consistently more frequent than those of *F. oxysporum*, both in epidermal and in outer cortical cells of inoculated roots. Occasional hyphae of *F. solani* were seen in the epidermal cells of the root 24 hr. after inoculation, and they were more frequent there after 48 hr. Four days after inoculation, the root surface had become discoloured and sections of these roots showed extensive colonization of the epidermal and outer cortical tissues. Many of the cells were packed with hyphae, and the cytoplasm of colonized cells was granular and stained deeply. Six days after inoculation, the discoloration of the root surface had extended and many of the epidermal cells were necrotic and collapsed, but the fungus had not penetrated further than the outer cortex.

No hyphae of *F. oxysporum* occurred in sections of roots 24 hr. after inoculation and they were rare after 48 hr., but after 4 and 6 days they were more frequent, although, unlike those of *F. solani*, they did not extensively colonize the host cells. The hyphae of *F. oxysporum* passed both inter- and intracellularly through the epidermis and cortex. The root surface was not discoloured, and the host cells remained intact, with no obvious reaction to invasion.

EFFECT OF INFECTION BY *FUSARIUM OXYSPORUM* AND *F. SOLANI* ON THE RESPIRATION INTENSITY OF PEA ROOTS

Examples of increased respiration rate as a host response to infection by fungi have often been reported (Allen, 1953) and include stem sections of cotton infected with *F. oxysporum* f. *vasinfectum* (Lakshmanan, 1955).

The following experiment was made to see whether disruption of host tissues by *F. solani* altered the respiration intensity of inoculated roots. Peas, variety Onward, were grown in beakers for 7 days, planted in moist unsterile loam, and then inoculated either with *F. oxysporum* or with *F. solani* by pouring 2 ml. of a centrifuged and re-suspended Czapek-Dox liquid culture of the fungus, 10 days old, over the root surface. Two ml. distilled water were poured over the root surface of control seedlings. After 3 days, the plants were carefully removed from the soil and the roots gently washed in running tap water, until no soil remained on them. A Warburg constant-volume type respirometer, as described by Dixon (1951), was used to measure the carbon dioxide output of the roots. Respiration intensity was defined as the volume (μ l.) of carbon dioxide evolved per hour, per gramme fresh weight. A total of about 10 cm. of root (5 $\times$ 2 cm. lengths), which gave an optimum reading on the manometer, was put into each vessel. Four vessels were used for each treatment, and they were put into a constant temperature water-bath at 25° C. and left open to the atmosphere for 30 min. to equilibrate. After the apparatus had been closed, readings were taken at 30 min. intervals for 3 hr. The fresh weight of the roots was determined immediately after the respiration intensity had been measured (Table 1).

Table 1. *Effect of F. oxysporum and of F. solani on the respiration intensity of pea roots*

		Respiration intensity (μ l. CO ₂ /hr./g. fresh weight)	Means
Control roots, replicates	1	345	369
	2	379	
	3	395	
	4	358	
Roots infected with <i>F. oxysporum</i> , replicates	1	446	412
	2	404	
	3	390	
	4	407	
Roots infected with <i>F. solani</i> , replicates	1	478	463
	2	453	
	3	488	
	4	431	

The respiration intensity of roots inoculated with *F. solani* was 25 % greater than that of the controls, and 12 % greater than that of roots inoculated with *F. oxysporum*. This effect cannot be attributed solely to the metabolic activity of the fungus, because the volume of mycelium was very small compared with that of the host tissue, so that most of the increased respiration must reflect a disturbance in the host's metabolism.

DISCUSSION

As *F. solani* did not inhibit *F. oxysporum* in artificial media, in the soil, or in the rhizosphere, the interaction responsible for the decrease in severity of wilt presumably occurs within the host tissues. Wilt symptoms are thought to be caused by enzymes or toxins produced by the pathogen in the vascular tissue and distributed from there in the transpiration stream (Gäumann, 1951; Dimond, 1955). If this is so, the severity of wilt might be correlated with the amount of toxin produced. My experiments show that the delayed onset of pea wilt occasioned by *F. solani* is accompanied by delayed colonization of the stele by *F. oxysporum*. *F. solani* apparently produces some biochemical or physical barrier that delays the progress of the wilt fungus towards the stele, and thereby delays the production of wilt toxins. Although many of the tests failed to show any inhibitor to *F. oxysporum* produced by *F. solani* in the host, some provided evidence for a chemical barrier in the form of a substance demonstrable in the extracts from stems with foot-rot lesions by its ability to inhibit germination of *F. oxysporum* spores.

The anatomical investigation showed that *F. solani* enters and colonizes the epidermis and cortex of pea roots more rapidly than does *F. oxysporum*, and that *F. solani* causes necrosis of the colonized cells and of those adjacent to them. Gäumann (1950) considers that necrosis of a plant cell is, in effect, a defence reaction that serves to localize a parasite, and the observed features of infection by *F. solani* f. *pisi* are in general agreement with this idea. However, the restriction of *F. solani* to the outer cortical

tissues and its very slow progress up the stem could be controlled by other factors, such as changes in the anatomical structure or in the nutrient status of the stem above the soil surface. If the defence-reaction hypothesis is correct, then a reaction initiated by *F. solani* in the cortex could also be effective against *F. oxysporum*, which has to pass through the cortex to reach the stele. Although there was no necrosis in the cells of stem sections until 4 days after inoculation with *F. solani*, earlier changes, not necessarily in structure, may have restricted *F. oxysporum* shortly after infection.

The facts that *F. oxysporum* is both slow to infect the host, and does not extensively colonize the cortical cells, indicate that the cortex is not a favourable environment for it. This fits Garrett's (1956) proposition that a wilt fungus is confined to the vascular tract 'by the active resistance to invasion of the living cells composing the surrounding tissue'. The development of a necrotic reaction within the cortex, before infection by *F. oxysporum*, could make that region even more unfavourable to this fungus and consequently hinder its progress to the stele.

The delay of *F. oxysporum* by *F. solani* may be entirely the result of physical damage, for Linford (1931) has shown that mechanical damage increases the resistance of pea roots to *F. oxysporum*. Similarly, Keyworth & Dimond (1952), working with tomato, found that roots injured in various ways, e.g. by hot water (30 sec. at 55 ° C.), formalin, hydrogen peroxide and heavy metal chloride salts, resisted infection by *F. oxysporum* f. *lycopersici* more than did uninjured roots.

A phenolic compound, which inhibited the germination of spores of *Aspergillus niger*, was obtained from the cut stems of broad beans (*Vicia faba*) by Spencer, Topps & Wain (1957), who considered that it was formed as a wound reaction when the stems were cut. The damage done to pea roots by *F. solani* is similar to that done to tomato and pea roots, and to bean stems, and it might increase the resistance of peas to wilt because of the presence of an inhibitory substance such as that extracted from the foot-rot lesions of peas. In addition, the injuries caused to plants by these earlier workers could be expected to increase respiration in the same way as *F. solani* does. Whatever the exact cause of the decrease in pea wilt in the presence of *F. solani*, it very probably arises from some change in the cortex of the host root, which delays the progress of *F. oxysporum* towards the vascular tissue.

The author wishes to thank the Agricultural Research Council for a Training Grant, and Dr E. W. Buxton for many helpful suggestions.

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(Accepted for publication 21 November 1958)

PROCEEDINGS, 1959

MEETINGS

16 January 1959. Meeting held at Birkbeck College, University of London, at 11.0 a.m. Vice-Presidents, Dr Mary Glynn and Dr C. J. Hickman, in the Chair.

D. H. LAPWOOD. 'Field resistance' and potato blight (*Phytophthora infestans*).

Breeders have been unable to produce potato varieties that remain immune from blight when grown on a large scale, because the fungus varies and soon produces forms able to attack the new varieties. Immunity has been based on the hypersensitive reaction of *Solanum demissum*, which is controlled by a few independent major genes. The alternative is a resistance based on many genes that retards the rate at which the haulm is destroyed. Varieties differ in this respect and those in which defoliation is slow are called 'field resistant'. An investigation was begun in 1956 to find the factors controlling the rate of destruction of King Edward (KE), Up-to-Date (UD), Majestic (MJ) and Arran Viking (AV), which show increasing haulm resistance in this order. Field experiments in 1956, 1957 and 1958 showed only small differences in the rate of defoliation of these varieties; at 50% defoliation, only 4-5 days separated the extremes. The progress of the disease was determined by the rates at which foliage was infected and by the rates at which infected foliage was destroyed.

Differences in the rate of infection of foliage depended on the number of spores produced rather than on the rapidity of the infection cycle or on the number of occasions when the fungus spread in the field. Sporulation differed from day to day, and depended on the variety, on the size of lesion and its position in the crop canopy. Differences between varieties were greater in susceptibility of stem and leaf petiole than of leaf lamina. UD and KE were particularly susceptible to axillary inoculation, whereas MJ and especially AV were resistant. Stem lesions were common in crops of the first two and rare in the others. The rate at which infected foliage was destroyed depended more on the position and number of lesions than on the rate the fungus spread within tissues. Infected petioles were quickly girdled and killed in UD and KE, but more slowly in AV. Such indirect destruction was relatively more important with varieties susceptible to axillary infection, and in years like 1957 when long intervals separated the occasions of spread.

J. R. COLEY-SMITH. Observations on the biology of sclerotia of *Sclerotium cepivorum* Berk.

An explanation has been sought for the occurrence of white rot on newly broken and rested land. The inability of *Sclerotium cepivorum* to survive in soil in the form of mycelium indicated the importance of sclerotia as perennating bodies. In the present investigation sclerotia have been produced by growing the fungus on sand-maizemeal slopes for 6 weeks at 20° C. A high proportion of such sclerotia persisted for 4 years under undisturbed field conditions at depths of 3, 6 and 9 in. below the soil surface. Similar high survival figures were obtained in pot experiments in which soil containing sclerotia was disturbed by weekly trowelling. It is clear that outbreaks of white rot on rested land could easily be due to survival of sclerotia, and so investigations were made of conditions favouring their germination. Sclerotia from 6-week-old cultures did not germinate in soil, but those recovered after burial in soil for a month or more did so readily. Germination of sclerotia was greatly favoured by presence of the host. Stimulation of sclerotial germination was demonstrated in a number of soils of widely differing pH, over a wide range of soil water content, in the presence of onion, leek, and shallot seedlings, and in the field. Amongst sclerotia placed on uninjured roots of onion seedlings in Petri dishes,

germination was highest at the root tip region. Root injury, whilst greatly augmenting sclerotial response, destroyed the positional effect.

That germination of sclerotia in soil was chemically induced was proved by the fact that: (i) contact with onion roots was unnecessary, (ii) it occurred in the presence of root extracts of onion and other *Allium* spp. No response of sclerotia occurred with root extracts of unrelated plants. In host range experiments, *S. cepivorum* was able to parasitize only members of the genus *Allium*. This effect is of adaptive significance, in that sclerotia are stimulated to germinate only by those plants which the fungus can parasitize.

W. G. KEYWORTH. Wirestem of brassica seedlings caused by *Rhizoctonia solani*.

Severe attacks of this disease in brassica seed-beds at the National Vegetable Research Station led to a search for methods of control that would be an improvement on the established treatment with mercuric chloride. This, although fairly effective, caused stunting of the plants. In view of the known effectiveness of pentachloronitrobenzene against *Rhizoctonia*, this compound was tested in glasshouse and frame trials against wirestem. It was applied either as a 20% powder or as a 75% wettable powder suspension, various doses of these materials being compared with each other and with mercuric chloride. PCNB proved to be highly effective, giving at least as good a control as mercuric chloride without the attendant dangers, and it should be of use in commercial practice.

R. B. MAUDE. Didymella stem and fruit rot of outdoor tomatoes.

Research on this subject was begun at the National Vegetable Research Station, Wellesbourne, in 1953, by Dr Dorothy E. Knight and continued by the writer in 1957-58. At the outset the topic was investigated under four headings: (i) sources of the disease and methods of control, (ii) studies on the pathogen, (iii) spread of disease and its control and (iv) resistance trials of *Lycopersicon* species and varieties. Infected propagating soil, field debris, and bamboo canes were shown to be potent sources of the disease. Internal and external seed infection was demonstrated, but in field trials no infected mature plants were obtained from this source. To control spread of the disease, a search was made during 1955-57 for effective fungicides of low mammalian toxicity which, when applied to the site of fungal attack, would prevent and/or eradicate infection. Laboratory screening tests were followed by field trials in which the fungicides were sprayed onto the stem bases, to test them for eradicator and protectant action. These trials demonstrated the effectiveness of Maneb (1 and 2% active ingredient) in both capacities. Captan (0.25 and 0.5% active ingredient) proved of protectant value only. Zineb was the least effective of the three. Field trials carried out in 1958 confirmed the usefulness of Maneb. Results using Captan were not quite as good as those previously obtained.

Resistance trials of wild species and varieties of *Lycopersicon* resulted in the selection of a highly resistant strain of *L. hirsutum*. This year the F_1 progeny of a cross between *L. hirsutum* and *L. esculentum* cv. Harbinger proved to be resistant.

M. J. W. WEBB. Studies on powdery mildews.

The effect of zinc on the powdery mildews of turnip and cabbage was studied, using plants growing in pots of soil in the glasshouse. The metal was added to the soil either as zinc sulphate or as zinc frit (powdered glass containing zinc oxide). It was absorbed by the plants and control of the mildews was obtained. In other trials, similar treatments with zinc had no effect on the powdery mildews of chrysanthemum, gooseberry and cucumber although zinc accumulated in the leaves. A laboratory screening test using leaf disks has been developed and has given indications that metals other than zinc may also inhibit powdery mildews.

7 March 1959. Meeting held at Birkbeck College, University of London, at 11.0 a.m. The President, Dr P. W. Brian, in the Chair.

PHYCOMYCETES

H. STENTON. Observations on Phycomycetes associated with the early colonization of the seed coats and roots of *Pisum sativum* L. by fungi.

See these *Transactions*, 41, 74-80, 1958.

M. W. DICK. Seasonal fluctuations in Saprolegniaceae.

Saprolegniaceae have been known to occur in soil since 1925, but no investigation has been carried out to determine whether Saprolegniaceae are ephemeral soil invaders or an integral part of the fungus flora of the soil. An account is given of a multiple-sample quadrat technique, suitable for sampling at regular intervals from a given area of soil, with a view to investigating this problem. The need for such a technique is demonstrated by the fact that while 1 sample may yield 1-2 or at most 6-7 species, 16 samples (the number per quadrat used in this investigation) frequently yield 7-8 species and may yield up to 12 different species, and it is possible to isolate 1 or 2 more than this using over 160 samples, but these additional species are relatively infrequent.

In this investigation samples were taken from the top 2 in. of soil including the litter layer, each sample being treated individually throughout. Species were recorded qualitatively (as presence = 1, absence = 0) for individual samples, the sum for 16 samples giving a quantitative estimate of abundance.

Eighteen quadrats from three different localities (Bedgebury Pinetum, Wimbledon Common and Domewood, near East Grinstead), including a number of different soil types, were sampled once a month for periods of 6-20 months, and in all cases it was found that these areas of soil contained a more or less constant flora of Saprolegniaceae.

Fluctuations in abundance from season to season have been observed in quadrats rich in Saprolegniaceae. These fluctuations appear to be the same in different quadrats from widely separated localities, and they are confirmed by statistical analysis. The observed fluctuations are independent of the relative frequencies of species constituting the flora, and the species recorded, for any one quadrat. All species tend to show the same fluctuations but differences of degree are found between different species. Spring and autumn appear to be periods of maximum abundance for these fungi.

P. ELIZABETH PERROTT. Observations on the ecology of some aquatic Phycomycetes.

Many commonly recorded species of aquatic Phycomycetes are able to grow on a wide variety of substrates and in waters which vary considerably in hydrogen-ion concentration. The results of the present investigation suggest that the 'rarity' of less commonly recorded species often appears to be due not simply to specificity of substrate and toleration of only a limited range of hydrogen-ion concentration, but also to temperature requirements and the time needed for the maturation of the resting spores. As a result of these last two factors, there is a distinct seasonal periodicity of growth, mainly restricted to spring and autumn.

These observations are based on the results of an investigation of the phycomycete flora of four groups of collecting stations, selected from a variety of freshwater habitats in North Staffordshire, Cheshire and South Wales. Regular monthly collections of a variety of substrates were made at each station, water temperatures were recorded daily, and the hydrogen-ion concentration of the water recorded each month, during the period October 1954 to December 1955. Supplementary collections, which confirm the original data, were made during 1956 and 1957.

The development of suitable methods of collection and subsequent treatment of material, which involved keeping substrates at 3° C., made it possible to obtain plentiful natural

growths of many rarely collected Phycomycetes. It was shown by experiment that at temperatures above 3° C. many of these delicate and often slow growing fungi are unable to compete with other organisms present in untreated pond water.

The forty-four species recorded belong to the following orders: Blastocladales, Monoblepharidales, Plasmodiophorales, Saprolegniales, Leptomitales, Lagenidiales and Peronosporales. The Chytridiales are excluded, and particular reference is made to members of the Monoblepharidales. The frequency of occurrence of species is indicated.

L. G. WILLOUGHBY. The quantitative assessment of spores of Saprolegniales occurring in natural bodies of fresh water.

In order to develop methods for the quantitative estimation of spores of Saprolegniales in samples of natural fresh water, the following procedures are being investigated: (i) baiting with complex organic substrata, (ii) plating with agar, (iii) membrane filtration. Most of the work so far has been concerned with procedure (ii), which has distinguished (a) a marked difference in spore concentration in different bodies of water sampled at the same time of year, (b) a marked difference in spore concentration in a single body of water sampled at different times of the year.

M. E. DAVIES. The physiology of *Phytophthora fragariae*.

See these *Transactions*, 42, 193-200, 1959.

P. K. C. AUSTWICK. The growth of mucoraceous fungi in animal tissue.

The mucoraceous fungi which are pathogenic to man and animals comprise seven species belonging to the genera *Mucor*, *Absidia* and *Rhizopus*. Most are known to occur saprophytically but can produce three types of disease in animals—the granulomatous type in which tumour-like growths are formed in the viscera, the ulcerative type chiefly affecting the mucous membrane of the digestive system and bovine mycotic abortion which appears to be a primary infection of the placenta. The appearance of the hyphae in tissues may be modified by the reaction of the animal to infection, so that specialized growth forms are produced in certain types of tissue. The morphology of these forms seems to be dependent upon the degree of reaction. Hyphae found in body fluids, e.g. allantoic fluid, are of the (a) saprophytic type and similar to those produced in liquid culture media with straight hyphae and frequent swellings. In granulomatous lesions, wide, frequently branching hyphae of the (b) parasitic type are produced and these are usually surrounded by giant cells. Ulcerative lesions contain the relatively little-branched saprophytic type of growth in the mucosa but intensely branched parasitic type in the submucosal tissues. Other structures including internal wall thickenings or pegs, septa and chlamydospores are also occasionally seen.

Material from seventy-six cases or outbreaks of disease associated with mucoraceous fungi has been examined during the last 7 years but less than a quarter gave isolates of the causal fungi. It is, therefore, important to attempt to define the morphological characters of the various species *in vivo*.

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